REGULATION OF UROKINASE PLASMINOGEN ACTIVATOR AND PLASMINOGEN ACTIVATOR INHIBITOR-1 IN MURINE BREAST CANCER CELLS

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^{o}C	Degrees celcius
$4T1^{\Delta PAR\text{-}1}$	4T1 cells expressing protease-activated receptor-1 shorthairpin RNA
$4T1^{\Delta PAR\text{-}2}$	4T1 cells expressing protease-activated receptor-2 shorthairpin RNA
$4T1^{GFP}$	4T1 cells expressing green fluorescent protein shorthairpin RNA
ANOVA	Analysis of variance
APC	Activated protein C
asTF	Alternatively spliced tissue factor
Avg	Average
BFA	Brefeldin A
BHK	Baby hamster kidney
Ca^{2+}	Calcium
$CD11b^{+}$	Cluster of differentiation-11b positive
CD14	Cluster of differentiation-14
CD142	Cluster of differentiation-142
CD45	Cluster of differentiation-45
cDNA	Complementary deoxyribonucleic acid
CHO	Chinese hamster ovary
cm^3	Cubic centimeters
CXCL-16	Chemokine (C-X-C motif) ligand 16
DAPI	4',6-diamidino-2-phenylindole
DTI	Direct thrombin inhibitor
ΔΔCt	Delta-delta cycle threshold

DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGFRvIII	Epidermal growth factor receptor variant III mutant
EGR1	Early growth response-1
ELISA	Enzyme-linked immunosorbent assay
ELK1	Ets-like gene-1
EPCR	Endothelial protein C receptor
EPR1	Effector cell protease receptor-1
ER	Estrogen receptor
ERK1/2	Extracellular signal-regulated kinase 1/2
et al.	Et alia
FBS	Fetal bovine serum
FII	Prothrombin
FIIa	Activated coagulation factor II/thrombin
FIII	Coagulation factor III/tissue factor
FITC	Fluorescein isothiocyanate
FIX	Coagulation factor IX
FIXa	Activated coagulation factor IX
FVa	Activated coagulation factor V
FVII	Coagulation factor VII

FVIIa	Activated coagulation factor VII
FVIIai	Active site-inhibited activated coagulation factor VII
FX	Coagulation factor X
FXa	Activated coagulation factor X
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCSF	Granulocyte colony-stimulating factor
GFP	Green fluorescent protein
GM-CSF	Granulocyte macrophage colony-stimulating factor
GPCR	G protein-coupled receptor
$GR1^+$	GR1 antigen positive
h	Hours
HCl	Hydrochloride
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HER-2	Human epidermal growth factor receptor-2
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
IACUC	Institutional animal care and use committee
IκBα	Inhibitory kappa B alpha
IL-1	Interleukin-1
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-12	Interleukin-12
KCl	Potassium chloride
kD	Kilodalton

K-ras	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
M	Molar
MAPK	Mitogen-activated protein kinase
M-CSF	Macrophage colony stimulating factor
MEK	Mitogen-activated protein kinase kinase
MEM	Minimal essential media
mFVIIa	Recombinant murine activated coagulation factor VII
$MgCl_2$	Magnesium chloride
min	Minute
μL	Microliter
μL	Milliliter
μM	Micromolar
mM	Millimolar
MMP-1	Matrix metalloproteinase-1
MMP-2	Matrix metalloproteinase-2
MMP-9	Matrix metalloproteinase-9
MMP-13	Matrix metalloproteinase-13
MMPs	Matrix metalloproteinases
mRNA	Messenger ribonucleic acid
NAB1	Nerve growth factor induced-A binding protein-1
NAB2	Nerve growth factor induced-A binding protein-2
NFκB	Nuclear factor kappa B
ng	Nanogram

nM	Nanomolar
NP-40	Nonyl phenoxypolyethoxylethanol
NRSA	National research service award
ns	Not significant
P	P value
PAI-1	Plasminogen activator inhibitor-1
PAI-2	Plasminogen activator inhibitor-2
PAR-1	Protease-activated receptor-1
PAR-2	Protease-activated receptor-2
PAR-3	Protease-activated receptor-3
PAR-4	Protease-activated receptor-4
PARs	Protease-activated receptors
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pELK1	Phosphorylated ets like gene-1
pERK1/2	Phosphorylated extracellular signal-regulated kinase 1/2
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PKCμ	Protein kinase C-mu
PLG	Plasminogen
PM	Plasmin
PMA	4-beta-phorbol-12 myristate 13-acetate
pPKCμ	Phosphorylated protein kinase C-mu

PTEN	Phosphatase and tensin homolog
PVDF	Polyvinylidene fluoride
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
rNAPc2	Recombinant nematode anticoagulant protein c2
rNAP5	Recombinant nematode anticoagulant protein 5
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SERPIN	Serine protease inhibitor
SFM	Serum-free media
shRNA	Short hairpin ribonucleic acid
siRNA	Small interfering RNA
SRE	Serum response element
SRF	Serum response factor
TAM	Tumor-associated macrophage
TAN	Tumor-associated neutrophil
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TGF-β	Transforming growth factor beta
TME	Tumor microenvironment
TNF-α	Tumor necrosis factor alpha
tPA	Tissue plasminogen activator
Tris	Tris(hydroxymethyl)aminomethane

TSP	Thrombospondin
U0126	1,4-Diamino-2,3-dicyano-1,4-*bis*(2-aminophenylthio)butadiene
UNC-CH	University of North Carolina at Chapel Hill
uPA	Urokinase plasminogen activator
uPAR	Urokinase plasminogen activator receptor
VEGF	Vascular endothelial growth factor
VTE	Venous thromboembolism
x g	Times gravity

CHAPTER I:

Introduction

BREAST CANCER

Carcinogenesis occurs due to a combination of genetic aberrations. For example, children who have inherited a loss of function mutation in the retinoblastoma gene (RB1) are predisposed to developing retinoblastoma earlier than children who have accumulated somatic RB1 mutations[1]. This finding was the basis for the Knudson two-hit hypothesis of tumorigenesis, which states that a loss of heterozygosity of a tumor suppressor gene predisposes these individuals to developing certain kinds of cancer. This hypothesis is widely accepted and is applicable many childhood and adult malignancies, including breast cancer[2]. In the year 2010, it is estimated that of the 1,529,560 newly diagnosed cancer cases in the United States, 209,060 (13.66%) of these cases will be breast cancers. These numbers translate into roughly 28% of women and 0.25% of men that are newly diagnosed with cancer[3]. Furthermore, 19.23% of female and 19.79% of male breast cancer patients are estimated to succumb to this disease[3]. Phenotypically, breast cancer is a genetically complex and heterogeneous disease and is therefore categorized according to the expression of the human epidermal growth factor receptor-2 (Her2/neu), estrogen receptor (ER), and progesterone receptor (PR). Breast cancers can be further stratified based on their gene expression profiles[4]. Treatment options for breast cancer patients include surgery, radiation therapy, chemotherapy, biopharmaceuticals, and hormone therapy[5]. In recent years, a substantial body of literature has accumulated suggesting that the tumor microenvironment, a combination of stromal cells, proteases, cytokines, and immune effector cells, also contributes to malignancy[6].

Proteases are indispensable for successful solid tumor growth and are abundant in the tumor microenvironment. Many of these enzymes degrade the extracellular matrix, which is

required for angiogenesis, local invasion, and distant metastasis[7]. Furthermore, extracellular matrix proteolysis increases tumor cell proliferation by liberating sequestered growth factors[8]. In this introductory chapter, I present an overview the coagulation protease cascade, the protease-activated receptor family, and the fibrinolytic system. In each chapter of this dissertation, I describe how these systems work in concert to promote tumor progression.

TISSUE FACTOR

Tissue factor (TF), also known as coagulation factor III (FIII), or cluster of differentiation 142 (CD142), is a 47kD transmembrane glycoprotein that is the cellular receptor for FVII/FVIIa[9]. The TF/FVIIa complex triggers the extrinsic coagulation cascade, otherwise known as the TF pathway (Figure 1.1). Briefly, the TF-FVIIa complex activates FX to FXa, which, in the presence of FVa, calcium, and phospholipids, forms the prothrombinase complex. The prothrombinase complex converts prothrombin to thrombin, which then cleaves fibrinogen to fibrin and activates platelets. TF-FVIIa also activates FIX to FIXa. The end result of this cascade is the generation of a clot composed of fibrin and platelets.

TF is overexpressed in a variety of cancers[10-12]. TF contributes to tumor angiogenesis as inhibition of TF reduces tumor vascularity *in vivo*[12-14]. Additionally, deletion of the TF cytoplasmic domain delays mammary tumorigenesis *in vivo*[15]. Despite these findings, the coagulation proteases generated as a result of the TF-FVIIa complex have a more established role in tumor progression via their ability to activate protease-activated receptors (PARs).

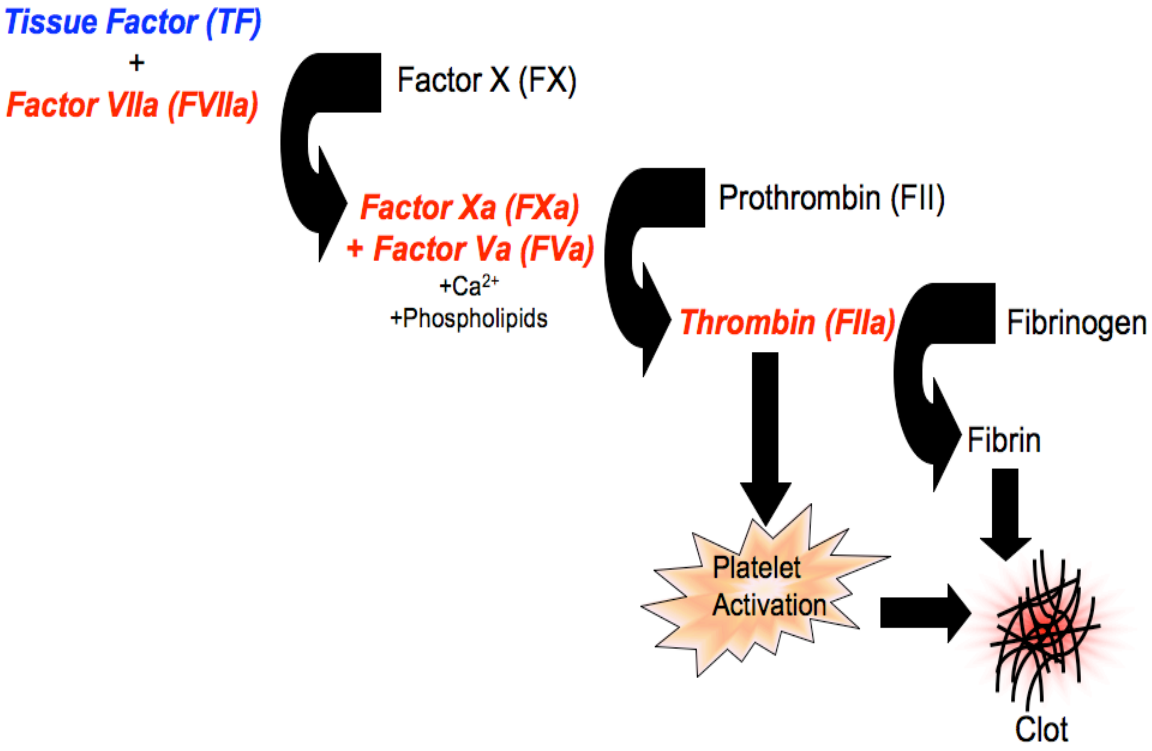

Figure 1.1: The extrinsic coagulation cascade. Tissue factor (TF) binds FVIIa on the cell surface, which then converts FX to FXa. The prothrombinase complex (FXa+FVa+Ca^{2+}+phospholipids) cleaves prothrombin to thrombin. Thrombin converts fibrinogen to fibrin in addition to activating platelets. Platelets and fibrin form a clot. TF-FVIIa also activates FIX to FIXa, which converts FX to FXa (not depicted). Blue represents a transmembrane receptor and red signifies an active protease

PROTEASE-ACTIVATED RECEPTORS

PARs are members of the G protein-coupled receptor (GPCR) family of which there are four[16]. PAR-1 and PAR-2 are expressed on a variety of cell types. Human endothelial cells express PAR-1, PAR-2, and PAR-3 whereas mouse endothelial cells express PAR-1, PAR-2, and PAR-4[17,18]. Human platelets express PAR-1 and PAR-4 while mouse platelets express PAR-3 and PAR-4. PARs are unique in that their ligand is attached to the receptor itself. Cleavage of the N-terminus of the receptor reveals this tethered ligand that then binds to the activation loop of the receptor to activate intracellular signaling pathways[17,19] (Figure 1.2). PARs are activated by a variety of proteases, including coagulation proteases (Table 1.1). In particular, FVIIa and FXa activate PAR-2 while FXa, and thrombin activate PAR-1[20].

Both PAR-1 and PAR-2 are overexpressed in breast cancer patient samples[21,22]. Activation of PAR-1 and/or PAR-2 confers an advantage to tumor cells *in vitro* and *in vivo*. For example, inhibition of PAR-1 decreased the survival, migration, and metastasis of breast tumor cells[23,24]. In similar fashion, PAR-2 activation increased breast cancer cell migration, invasion, and expression of vascular endothelial growth factor *in vitro*, and inhibition of PAR-2 decreased tumor angiogenesis *in vivo*[25-28].

FIBRINOLYSIS

Fibrinolysis, the proteolytic dissolution of fibrin clots, is controlled by the plasminogen activator system. The key components of this system includes the serine proteases tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA), the urokinase plasminogen activator receptor (uPAR), and plasminogen. In short, tPA released

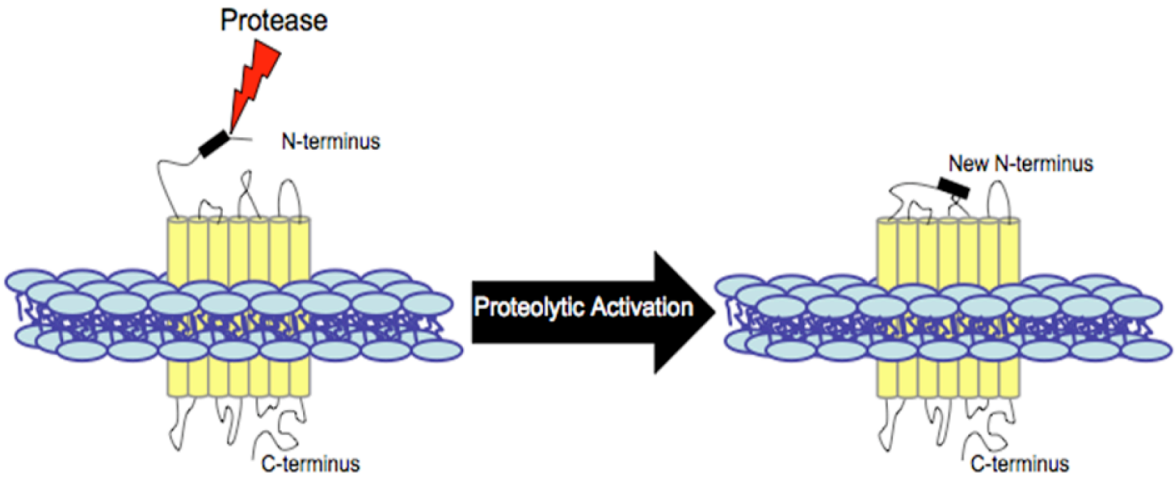

Figure 1.2: Mechanism of proteolytic activation of PAR-1 and PAR-2. A protease (red) cleaves the N-terminus of the receptor proximal to the tethered ligand sequence (black box). The new N-terminus interacts with the activation loop of the receptor to enable downstream signaling.

Table 1.1: Proteolytic PAR agonists

PAR-1	Thrombin FXa APC Plasmin MMP-1 Granzyme A
PAR-2	Trypsin Tryptase FVIIa FXa Matriptase Granzyme A Kallikreins
PAR-3	Thrombin
PAR-4	Thrombin Trypsin FVIIa FXa Plasmin Cathepsin G Kallikreins

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locally from endothelial cells and the cell surface bound uPA/uPAR complex efficiently convert plasminogen to the active serine protease plasmin which then degrades fibrin[29]. The serine protease inhibitor (SERPIN), plasminogen activator inhibitor-1 (PAI-1), negativelyregulates fibrinolysis by binding to the active sites of tPA and uPA, thus inhibiting their proteolytic function. Additionally, PAI-2 also inhibits tPA and uPA. However, this SERPIN is present at extremely low levels in plasma[29].

uPA is overexpressed in breast cancers and these elevated levels correlate with decreased relapse free survival and decreased overall survival rates in patients with invasive breast cancer[30,31]. *In vitro*, uPA induces proliferation and invasion of breast cancer cells[32,33]. Additionally, overexpression of uPA increases breast cancer metastasis whereas reducing uPA decreases metastasis[34-36]. Furthermore, in $HER2^{+}$ breast cancer patient samples, elevated uPA levels correlate with increased metastasis[37]. Moreover, inhibition of uPA and/or uPAR impaired endothelial cell tube formation and increased apoptosis of breast cancer cells *in vitro*[38]. uPA bound to uPAR also provides localized proteolysis via cell surface plasmin generation. In fact, plasmin activates matrix metalloproteinases (MMPs), which, in addition to plasmin itself, degrade the extracellular matrix, favoring cell invasion[39,40].

Although PAI-1 inhibits uPA function, paradoxically, PAI-1 also promotes malignancy (Figure 1.3). For example, elevated PAI-1 levels in breast cancer patients are correlated with poor patient outcome[30,31]. Moreover, elevations of PAI-1 correlate with increased metastasis in breast cancer patients[41]. Similar to uPA, PAI-1 contributes to tumor angiogenesis and metastasis *in vivo*[42,43]. Increased PAI-1 levels correlated with an increased

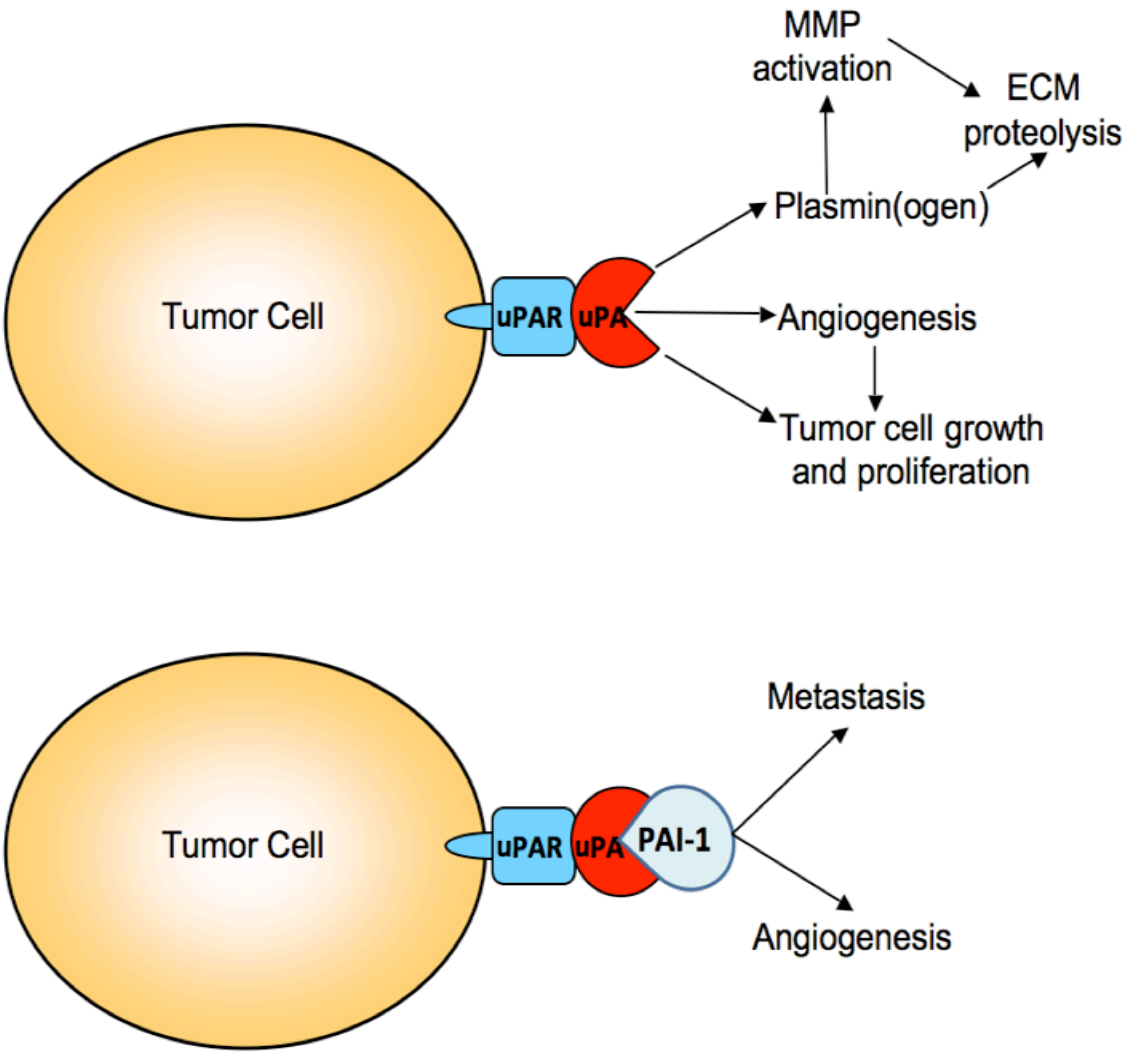

Figure 1.3: uPA and its inhibitor, PAI-1, promote tumor progression. Cell surface uPA has roles in tumor angiogenesis and tumor growth. uPA converts plasminogen to plasmin thereby creating a zone of localized proteolysis (top). Plasmin activity favors tumor cell invasion via matrix degradation and activation of MMPs. PAI-1 inhibits uPA function, but in doing so promotes tumor angiogenesis and metastasis (bottom).

degree of vascular remodeling in clinical breast tumor samples[44]. Additionally, inhibition of PAI-1 decreased *in vitro* migration of breast cancer cells[45].

Chapter 2 is a review the role of TF in cancer, focusing on the cellular distribution of TF and how this is relevant to tumor progression. More specifically, I discuss TF expression in cancer cells and in non-malignant host cells, the contribution of TF-dependent PAR signaling and how this promotes tumor angiogenesis, tumor growth, and tumor metastasis. This review, "Tissue Factor Expression by Malignant Cells Contributes to Tumor Progression", was recently published in the *Journal of Coagulation Disorders*.

Chapter 3, entitled "Protease-Activated Receptors Mediate Cross-Talk Between Coagulation and Fibrinolysis", was recently published as an original research article in *Blood*. This body of work demonstrates that uPA is induced in response to the coagulation proteases FXa and thrombin via PAR-1 in 4T1 metastatic mouse breast cancer cells. Furthermore, I discovered that there are intracellular stores of uPA associated with the Golgi apparatus and that these stores are rapidly released upon PAR-1 activation. Additionally, a novel PAR-dependent PAI-1 regulatory mechanism was revealed. In short, transactivation of PAR-2 by thrombin-activated PAR-1 resulted in PAI-1 mRNA and protein expression.

In Chapter 4, I further explore the PAI-1 regulatory mechanism discovered in Chapter 3. I found that activation of the PAR-1/PAR-2 complex activated the p42/p44 mitogen activated protein kinase (MAPK) signaling cascade and induced the phosphorylation of ets-like gene 1 (ELK1), which increases the expression of the transcription factor early growth response-1 (EGR1), a known positive regulator of PAI-1 gene expression. Furthermore, the p42/p44 MAPK pathway was necessary for PAI-1 protein expression. Interestingly, I also

discovered that FXa induced p42/p44 MAPK intracellular signaling but did not result in increased EGR1 mRNA or protein expression.

Chapter 5 is the discussion of the entirety of the experimental data presented in this dissertation. Here, I present a model that illustrates how this data adds to the existing literature regarding breast cancer invasion and tumor angiogenesis. Additionally, I address the limitations of my work and provide experimental suggestions to further continue the investigation of how PAR-1 and PAR-2 contribute to breast tumor progression.

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CHAPTER II:

Tissue Factor Expression by Malignant Cells Contributes to Tumor Progression

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ABSTRACT

Tissue factor (TF) is a transmembrane protein that binds its ligand factor VII/VIIa (FVII/FVIIa) and initiates the coagulation protease cascade. TF is essential for hemostasis. In addition, TF has been implicated in several pathological processes, including venous thromboembolism, sepsis, inflammation, and cancer. Aside from their primary roles in coagulation, FVIIa and the downstream proteases FXa and FIIa (thrombin) can also cleave and activate protease-activated receptors (PARs) on cells to induce intracellular signaling. In this review, we will summarize the proposed mechanisms by which TF contributes to tumor progression. Specifically, we will focus on how TF expression by cancer cells and host cells promotes malignancy by increasing angiogenesis, tumor growth, and metastasis.

INTRODUCTION

There has been a longstanding association between malignancy and coagulation. This dates back to initial reports of increased hypercoaguability in cancer patients described by Armand Trousseau in the mid-19th century[1]. Today, cancer associated thrombosis is a clinically relevant issue. In a population-based case-control study, approximately 20% of all patients diagnosed with venous thromboembolism (VTE) were also diagnosed with a malignancy[2]. In a large cohort study, Blom and colleagues reported that the risk of VTE in cancer patients is over 10 times greater than that of the cancer-free population[3]. This risk was further increased in patients with distant metastasis or receiving chemotherapy. An independent study reported that cancer patients had a 4-fold greater VTE risk in comparison to those without cancer[4]. Tissue factor (TF) is a member of the class 2 cytokine receptor family and serves as the transmembrane receptor for coagulation factor VII (FVII)[5].

Activated FVII (FVIIa) bound to TF initiates the coagulation protease cascade, which includes the proteases FXa, FVIIa, and FIIa (thrombin), resulting in the formation of a fibrin clot[6,7].

There is a significant correlation between the incidence of VTE and TF levels in cancer patients[8-12]. Furthermore, TF is expressed in a variety of malignancies, most notably in breast, brain, colorectal, lung, renal cell, ovarian, prostate, hepatocellular, and pancreatic cancers[13]. According to the American Cancer Society, it is estimated that, when combined, these cancers will account for roughly 59% of all newly diagnosed cases in the United States in 2010[14]. Furthermore, elevated levels of TF correlate with a reduced disease-free survival and overall survival rate[15-17]. This may reflect the fact that increased TF levels coincide with tumor aggressiveness both *in vitro* and *in vivo*. Recent studies have attempted to understand the mechanisms linking TF expression and tumor progression. This review will focus on TF-dependent activation of coagulation on the tumor cell surface and how this contributes to tumor progression via signaling through protease-activated receptors (PARs).

TF EXPRESSION BY CANCER CELLS

Oncogene activation and loss of tumor suppressor genes, two genetic insults required for carcinogenesis, increase TF expression in transformed cells. For instance, oncogenic activation of v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (K-ras), epidermal growth factor (EGFR), mutant EGFR (EGFRvIII), human epidermal growth factor receptor-2 (HER-2), as well as loss of the tumor suppressor genes phosphatase and tensin homolog (PTEN) and p53, all increase TF expression in a variety of cancer cells[18-21]. A recent report indicated that ectopic expression of EGFRvIII in human glioblastoma cells induced the

expression of TF, PAR-1, PAR-2, and FVII[22]. This equips these tumor cells with the necessary components for TF-dependent PAR signaling. Furthermore, pharmacological inhibition of the oncoproteins EGFR and HER-2 diminished TF expression in human squamous cell carcinoma and human breast carcinoma cell lines, respectively[18].

The tumor microenvironment also modulates TF expression in tumor cells. There is an abundance of inflammatory cytokines and chemokines present within the tumor stroma, many of which increase the activation of the nuclear factor-kappa B (NF-κB) signal transduction pathway[23,24]. Interestingly, tumor cells have high levels of NF-κB transcription factor activity[25-28], which may contribute to the high TF expression in tumor cells as previous studies have shown that inducible TF gene transcription in monocytes and endothelial cells is controlled, in large part, by NF-κB[29-31].

Interestingly, statins have been shown to attenuate NF-κB signaling[32,33]. *In vitro,* simvastatin inhibited nuclear translocation of NF-κB and decreased TF mRNA expression and TF-dependent signaling in tumor cells *in vitro*[34]. TF has a 100 amino acid domain that is structurally related to cytokine receptors leading to its classification as a member of the class 2 cytokine receptor family[5]. In addition to NF-κB, the TF promoter is also regulated by the transcription factor early growth response-1 (EGR1)[35]. Brat and colleagues have shown that hypoxia positively regulates tumor cell TF expression and activity by inducing the expression EGR1[36,37].

TF expression is not restricted to the cell surface as tumors are capable of shedding TF-positive microvesicles, also called microparticles[8,38]. *In vitro*, ovarian cancer cells have been shown to release TF-positive microparticles[39]. Our laboratory has demonstrated that tumor cells are a major source of TF-positive microparticles *in vivo* in a human pancreatic

cancer xenograft mouse model (Wang and Mackman, unpublished data). Moreover, murine tumor cells release TF-positive microparticles into the circulation[40]. In addition, an alternatively spliced form of TF (asTF) lacking both the transmembrane and cytoplasmic domains has been identified[41]. asTF is released from cultured pancreatic tumor cells and has been reported to promote the growth and angiogenesis of pancreatic tumors *in vivo*[42,43]. Additional TF splice variants have also been identified in tumor cells, although their contribution to tumor progression has yet to be established[44]. We, and others, have also shown that TF-positive microparticles are increased in cancer patients[9-11,45]. It is evident that the aberrant expression of cancer cell TF is regulated at multiple levels, reflecting the complexity and diversity of neoplastic cells.

TF EXPRESSION BY NON-MALIGNANT HOST CELLS

Tumors are often referred to as "wounds that do not heal" highlighting the potent inflammatory response and subsequent tissue remodeling that is inherent in both of these processes[46]. As discussed earlier, tumors are a major source of inflammatory mediators, including tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), and IL-6[23,24]. Both TNF-α and IL-1 induce TF expression in cultured endothelial cells[29,47,48]. Additionally, activation of endothelial cell PAR-1 has been shown to induce TF expression in these cells[49]. An additional component of the heterogeneous cell population that comprises solid tumors are fibroblasts[50,51]. Fibroblasts express high levels of TF and this expression can be further increased by transforming growth factor-beta (TGF-β), which is present within the tumor stroma[52]. Additionally, activated macrophages and neutrophils, both of which express TF, comprise a significant portion of the solid tumor mass[53-56].

Activated monocytes and macrophages are also a source of TF-positive microparticles [57,58]. These microparticles allow transfer of TF to a variety of cells. A clinical case study on plasma from a patient with giant-cell lung carcinoma observed circulating TF-positive microparticles bearing the monocyte marker CD14[59]. A second study of colorectal cancer patients found that the circulating TF-positive microparticles in the patient plasma samples were positive for either CD14 or the leukocyte common antigen CD45[60]. In addition to docking to tumor cells, it was demonstrated that monocyte-derived TF-positive microparticles fused with the membranes of endothelial cells and neutrophils, thereby conferring a procoagulant phenotype on these non-malignant host cells[61,62].

TF AND COAGULATION PROTEASES INCREASE TUMOR ANGIOGENESIS

TF expression is essential for hemostasis and proper blood vessel development[63]. Additionally, localized activation of coagulation on endothelial cells may contribute to angiogenic signaling to repair damaged vessels[64]. TF has also been implicated in tumor angiogenesis. TF was localized to the vascular endothelium within tumors samples of patients with invasive breast cancers but not in samples from patients with benign fibrocystic disease[65]. However, Luther *et al.* reported that TF is indiscriminately expressed in both benign and malignant mammary tissue growths[66]. Despite the controversy, recent publications do suggest that the expression of tumor cell TF is associated with tumor angiogenesis. For example, overexpression of tumor cell TF increased tumor vascularization *in vivo* and increased vascular endothelial growth factor (VEGF) expression as well as decreased the expression of the anti-angiogenic molecule thrombospondin (TSP) *in vitro*[67]. Conversely, silencing TF in Meth-A sarcoma or colorectal cancer cells resulted in decreased

tumor angiogenesis, decreased VEGF expression, and increased TSP expression[21,67]. Additionally, an anti-TF antibody reduced tumor vascularity and VEGF expression in human squamous carcinoma cells *in vivo*[20]. In clinical prostate cancer samples, TF expression correlates with VEGF expression, further suggesting a role for TF in tumor angiogenesis[68].

PARs are members of the seven membrane-spanning G-protein coupled receptor family[69]. These receptors are proteolytically activated by a variety of different proteases, including coagulation proteases[70]. For instance, FVIIa and FXa cleave PAR-2 to induce intracellular signaling, while FXa and thrombin cleave PAR-1 (Figure 2.1)[70,71]. Interestingly, PAR-1 and PAR-2 are upregulated in pancreatic cancer cells and are thought to be a part of the "angiogenic switch"[72]. Treatment of the MDA-MB-231 human breast cancer cell line with FVIIa, FXa, or thrombin induced IL-8 expression *in vitro*[73,74]. In a separate study, IL-8 promoted endothelial cell survival, proliferation, and secretion of matrix remodeling proteases, all of which are required for angiogenesis[75]. Together, this suggests that tumor-derived IL-8 may function as a pro-angiogenic stimulus for endothelial cells.

In certain tumors, host-derived TF also contributes to tumor angiogenesis as exemplified by the finding that TF expressing tumors grown in mice expressing low levels of TF have smaller blood vessels compared with controls[76]. Fibroblasts have been shown to secrete VEGF in a TF-FVIIa-FXa and thrombin-dependent manner[77]. As mentioned earlier treatment of tumor cells with coagulation proteases induced the expression of a variety of chemokines, thereby enhancing leukocyte recruitment into the tumor stroma. Once recruited into the tumor stroma, tumor associated macrophages (TAMs) and tumor associated neutrophils (TANs) further enhance angiogenesis[78-80]. In fact, a recent study has shown that a lack of PAR-2 and/or the lack of the cytoplasmic domain of TF resulted in a

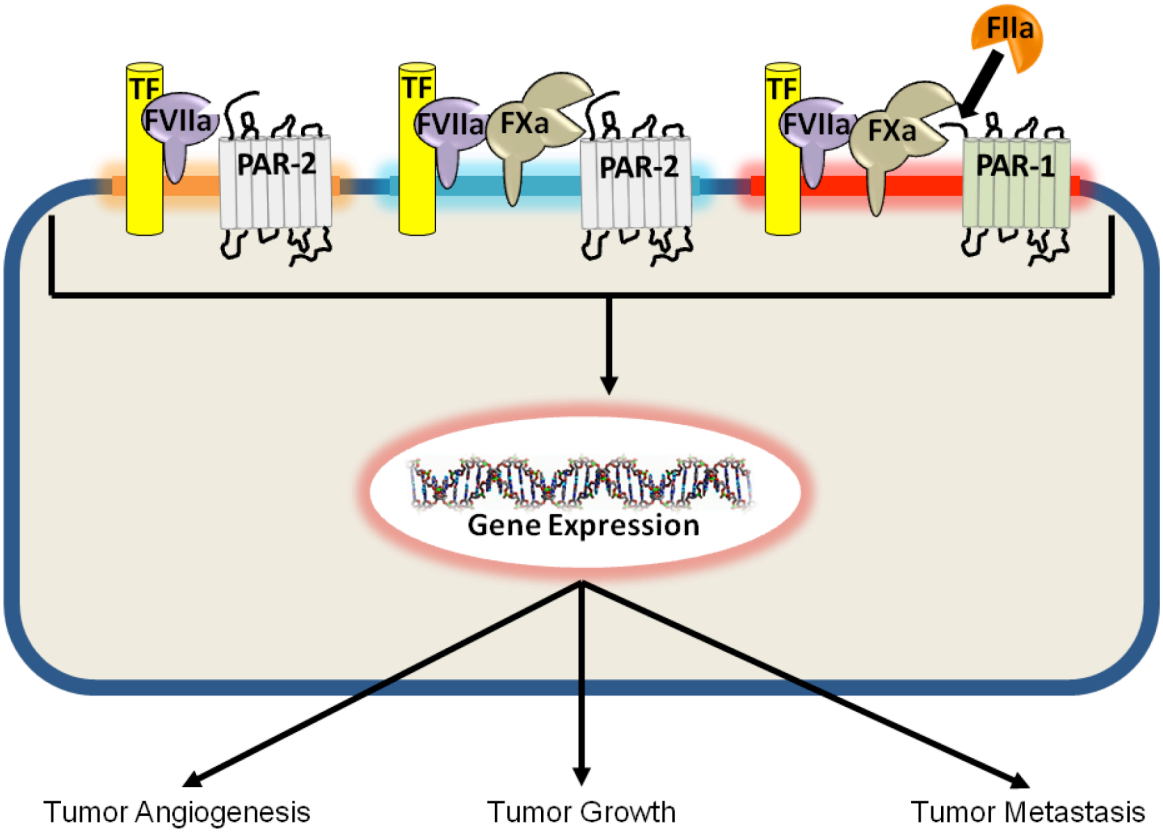

Figure 2.1: Tissue factor-dependent activation of PARs contributes to tumor progression. TF expressed on the tumor cell surface activates the coagulation cascade once bound to factor VIIa (FVIIa). Intracellular signaling is induced when the TF-FVIIa complex or FXa cleave PAR-2. Activation of PAR-1 by FXa or thrombin will also induce signaling. PAR activation results in the expression of genes that promote tumor growth, tumor angiogenesis, and tumor metastasis.

reduction of TAMs and decreased vascular density in a polyoma middle-T breast cancer model[81].

TF AND COAGULATION PROTEASES PROMOTE TUMOR GROWTH

Experimental evidence reveals that TF influences tumor growth. One mechanism by which TF and coagulation proteases may contribute to tumor growth is via PAR-1 dependent inhibition of apoptosis. Incubation of human breast tumor cells with either FVIIa and FX or FXa resulted in a significant decrease in apoptosis of serum-starved tumor cells[82]. Furthermore, TF-FVIIa and TF-FVIIa-FXa signaling also decreased apoptosis in baby hamster kidney (BHK) cells[83].

Overexpression of TF increased the growth of Meth-A sarcoma tumors *in vivo* whereas reduced TF expression was associated with decreased tumor growth[67]. Likewise, reducing TF expression in colorectal cancer cells resulted in decreased tumor volume *in vivo*[21]. Furthermore, inhibition of TF substantially delayed the initial onset of human epithelial carcinoma growth *in vivo*[20]. Conversely, overexpression of TF in a TF-deficient human pancreatic cell line dramatically increased the growth rate of the implanted tumors[84].

Inhibiting different steps in the coagulation pathway with anticoagulants has also been explored as a means of reducing tumor growth. In an attempt to determine which component of the TF pathway is responsible for the growth of certain tumors *in vivo*, TF-FVIIa was inhibited with recombinant nematode anticoagulant protein c2 (rNAPc2) while FXa was inhibited with rNAP5, a nematode anticoagulant protein specific for FXa[85]. rNAPc2 reduced the growth of melanoma cells whereas rNAP5 did not, suggesting that the TF-FVIIa complex contributes to tumor growth of melanoma cells. In a separate study,

rNAPc2 reduced the mitotic index, tumor weight, and tumor volume of colorectal cancer xenografts[86]. Similarly, Ixolaris, a tick anticoagulant protein that inhibits the TF-FVIIa complex, reduced the growth of glioblastoma xenografts[87].

Other tumors may utilize different mechanisms downstream of TF-FVIIa to confer a growth advantage. For instance, thrombin was recently proposed to be a tumor growth factor[88]. Indeed, one study revealed that thrombin stimulation induced cell cycle activation of prostate cancer cells[89]. However, thrombin inhibition appears to be tumor specific as hirudin inhibited B16 melanoma growth *in vivo* but not the growth of K1735 melanoma or colon cancer cells[90].

TF-DEPENDENT ACTIVATION OF COAGULATION PROMOTES TUMOR CELL INVASION AND METASTASIS

The presence of TF initiates the local generation of coagulation proteases on the tumor cell surface[91]. *In vitro*, FVIIa, FXa, and thrombin activate PARs on tumor cells to promote migration and invasion[73,92-94]. Ectopic TF expression in a human pancreatic cancer cell line increased *in vitro* tumor cell invasion[84]. In glioma cells, inhibition of TF decreased tumor cell migration and invasion *in vitro*[95]. Mueller and Ruf demonstrated that TF expression in Chinese hamster ovary (CHO) cells enhances experimental hematogenous metastasis[96]. The candidate mechanism by which TF-dependent PAR activation enhances tumor invasion is likely via increased extracellular matrix proteolysis. Treatment of breast cancer cells with FVIIa, FXa, or thrombin increased the release of urokinase plasminogen activator (uPA) and plasminogen activator inhibitor-1 (PAI-1) from the cells in a PAR-dependent manner[74,97,98]. Both uPA and PAI-1 contribute to tumor cell invasion and

metastasis *in vitro* and *in vivo*[99-101]. Thrombin-dependent signaling also promotes extracellular matrix degradation via expression of matrix metalloproteinases (MMPs). Thrombin increased *in vitro* invasion via the expression and release of MMP-9 and the expression of β1-integrin in osteosarcoma cells, as well as via increased MMP-2 and MMP-13 expression in human chondrosarcoma cells[102,103]. Furthermore, thrombin induced the expression of the protease cathepsin-D in murine breast cancer cells, which coincided with increased invasion[104].

Inhibition of TF with an anti-TF inhibitory antibody (5G9) or covalently inactivated FVIIa (FVIIai) reduced the number of experimental metastatic foci in the lungs of mice[96]. Likewise, inhibition of TF decreased melanoma metastases to the lung[105]. Tissue factor pathway inhibitor (TFPI), the endogenous inhibitor of TF, also reduced the experimental metastasis of melanoma cells[106]. Additionally, pharmacological inhibition of TF reduced metastasis in an *in vivo* model of colorectal cancer[86]. The current hypothesis is that TF expression by tumor cells leads to fibrin coating of the cells that reduces natural killer cell anti-tumor activity and promotes tumor cell adhesion to the endothelium, thus promoting hematogenous metastasis[107-109].

TF-FVIIa-PAR2 signaling induced the expression of IL-8, granulocyte-macrophage colony stimulating factor, (GM-CSF), and macrophage colony stimulating factor (M-CSF) in a variety of breast cancer cell lines[73,74]. These chemokines recruit leukocytes into the tumor stroma. Once recruited, these leukocytes may become TAMs or TANs, thereby promoting tumor invasion and metastasis[110-113]. In fact, the presence of thrombin within the tumor stroma may facilitate this process as thrombin stimulated macrophages adopted a TAM phenotype, resulting in increased invasion of ovarian cancer cells[114].

CONCLUSION

There is a substantial amount of data implicating TF, coagulation proteases, and PARs in tumor angiogenesis, growth, and metastasis. Despite these advances, several questions still remain. It is generally accepted that tumors are the major source of TF in malignancy. However, at the present it is unclear how TF expression by non-malignant host cells within the tumor stroma contributes to tumor progression. What is the functional relevance of TF-positive microparticles in tumor progression? Can TF be used as a reliable biomarker for malignancy? Overall, TF is an attractive target for anti-caner therapy. Further studies are needed to determine which specific targets of the TF pathway are the most efficacious and whether the potential hemorrhagic complications of targeting TF or thrombin are worth the risk in comparison to targeting PARs.

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CHAPTER III:

Protease-Activated Receptors Mediate Crosstalk Between Coagulation and Fibrinolysis

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ABSTRACT

The coagulation and fibrinolytic systems contribute to malignancy by increasing angiogenesis, tumor growth, tumor invasion, and tumor metastasis. Oncogenic transformation increases the expression of tissue factor (TF) that results in local generation of coagulation proteases and activation of protease-activated receptor (PAR)-1 and PAR-2. We compared the PAR-dependent expression of urokinase plasminogen activator (uPA) and plasminogen activator inhibitor (PAI)-1 in two murine mammary adencocarcinoma cell lines: metastatic 4T1 cells and non-metastatic 67NR cells. 4T1 cells expressed TF, PAR-1 and PAR-2 whereas 67NR cells expressed TF and PAR-1. We also silenced PAR-1 or PAR-2 expression in the 4T1 cells. We discovered 2 distinct mechanisms for PAR-dependent expression of uPA and PAI-1. First, we found that factor Xa or thrombin activation of PAR-1 led to a rapid release of stored intracellular uPA into the culture supernatant. Second, thrombin transactivation of a PAR-1/PAR-2 complex resulted in increases in PAI-1 mRNA and protein expression. Cells lacking PAR-2 failed to express PAI-1 in response to thrombin and factor Xa did not activate the PAR-1/PAR-2 complex. Our results reveal how PAR-1 and PAR-2 on tumor cells mediate cross-talk between coagulation and fibrinolysis.

INTRODUCTION

Tissue factor (TF) is the cell surface receptor for coagulation factor VII/VIIa (FVII/VIIa). TF is expressed on various cell types and its expression is upregulated by oncogenic transformation, conferring a procoagulant phenotype to cancer cells[1-4]. Tumor cell TF locally activates the coagulation cascade when clotting factors in the blood enter the stroma from leaky tumor vasculature. Indeed, coagulation proteases, such as factor VIIa

(FVIIa), factor Xa (FXa), and thrombin, have been shown to contribute to tumor proliferation, migration, and angiogenesis[5-8].

The mechanism by which coagulation proteases exert their tumor enhancing effects is, in part, via activation of protease-activated receptor (PAR)-1 and PAR-2[9-12]. PARs belong to a family of G-protein coupled receptors that are proteolytically activated by a variety of proteases. FXa and thrombin activate PAR-1, whereas FVIIa and FXa activate PAR-2[9,13,14]. In addition, PAR-1 can be activated by matrix metalloproteinase-1, plasmin, and activated protein C bound to endothelial protein C receptor (APC-EPCR)[13,15-17]. Trypsin, tryptase, kallikreins, and matriptase activate PAR-2 (for review, see Trejo[18]). Furthermore, PARs can transactivate one another. For instance, thrombin can bind to the N-terminus of PAR-3, which acts as a cofactor that allows the protease to activate PAR-4 and induce intracellular signaling in mouse platelets[19]. In human platelets PAR-1 and PAR-4 both signal. In addition, PAR-1 forms a heterodimer with PAR-4 to initiate signaling in response to thrombin[20]. Furthermore, O'Brien and colleagues demonstrated, using pharmacological inhibitors of PAR-1 and a mutant PAR-1 incapable of signaling, that thrombin cleaved PAR-1 can transactivate PAR-2 in human endothelial cells and transfected COS-7 cells[21].

Urokinase plasminogen activator (uPA) is a serine protease that converts plasminogen to plasmin. Plasminogen activator inhibitor (PAI)-1 is the endogenous inhibitor of uPA that forms a heterotrimeric complex with uPA and the uPA receptor (uPAR)[22]. Aside from their roles in regulating fibrinolysis, both uPA and PAI-1 promote metastasis[22-25]. Furthermore, uPA and PAI-1 are also involved in angiogenesis and endothelial cell migration[26-28]. Increased levels of uPA and PAI-1 are found in breast cancer patients, both correlating with poor prognosis and reduced survival[29,30]. Proteolytic activation of PAR-2 by FVIIa bound to

TF increases uPA, PAI-1, and uPAR mRNA expression in human breast cancer and pancreatic cancer cell lines[31,32]. PAR-1 and PAR-2 agonist peptides also increase both uPA and PAI-1 mRNA levels in the MDA-MD-231 human breast cancer cell line[31].

The aim of this study was to determine the role of PAR-1 and PAR-2 in the crosstalk between coagulation proteases and the regulation of plasmin generation in breast cancer cells. We used nonmetastatic (67NR) and metastatic (4T1) murine mammary adenocarcinoma cell lines because TF, coagulation proteases, uPA, and PAI-1 have been shown to contribute to metastasis. Here we describe 2 different mechanisms by which FXa and thrombin regulate uPA release and PAI-1 mRNA expression.

MATERIALS AND METHODS

Reagents.

Recombinant mouse FVIIa (mFVIIa) was provided by Dr Lars Petersen (Novo Nordisk). Purified human factor X (FX), FXa, and α-thrombin were obtained from Haematologic Technologies Inc. Puromycin dihydrochloride was obtained from Mediatech. Brefeldin A (BFA) was purchased from BD Biosciences. Dimethyl sulfoxide, Triton X-100, DAPI (4,6-diamidino-2-phenylindole), and dithiothreitol were obtained from Sigma-Aldrich. 4-β-phorbol-12-myristate 13-acetate (PMA) was purchased from Cell Signaling Technologies. Complete protease inhibitor cocktail tablets and phosphatase inhibitor cocktail were purchased from Roche.

Cell culture.

67NR and 4T1 mouse mammary adenocarcinoma cell lines were obtained from Dr.

Fred Miller (Michigan Cancer Foundation). The 67NR and 4T1 cells were derived from the same spontaneously arising primary tumor and therefore represent the varied metastatic potential that exists in cells within a single tumor[33]. Importantly, the 4T1 cell line spontaneously metastasizes to the lymph nodes, lung, liver, bone, and brain and is therefore regarded as a clinically relevant breast tumor model that closely recapitulates human stage IV metastatic disease[34]. Conversely, the 67NR cell line is nonmetastatic. Cells were maintained in mimimum essential medium-alpha (Gibco), with 10% fetal bovine serum (Omega Scientific), and 1% penicillin/streptomycin (Sigma-Aldrich). Before treatment with coagulation factors or BFA, cells were grown to confluence in 12-well plates (Corning Inc.) and serum-starved overnight in serum-free media (SFM). The following coagulation proteases or zymogens were diluted in SFM and used to treat the cells: recombinant mFVIIa, human zymogen FX, mFVIIa and FX, human FXa, and human α-thrombin. PMA was diluted in SFM and incubated with the cells for 1 hour. BFA or dimethyl sulfoxide vehicle control were diluted in SFM and cells were pre-incubated for 3 hours before the addition of coagulation proteases or zymogen.

Short hairpin RNA.

Lentiviral particles carrying plasmids encoding short hairpin RNA (shRNA) to either mouse PAR-1 or mouse PAR-2 were obtained from the University of North Carolina at Chapel Hill (UNC-CH) shRNA Core Facility. The GFPshRNA control plasmid (Addgene plasmid 12273) was obtained from Addgene, and packaged into lentiviral particles by the UNC-CH shRNA Core Facility[35]. Cells were transduced using Viraductin (Cell Biolabs) according to manufacturers protocol with an additional spin inoculation at 1,250 x *g* for 90

minutes at 22°C before placing the cells in the incubator. Twenty-four hours posttransduction, 12μg/mL of puromycin was added to the culture media to select stable cell populations containing the lentivirus. Entire populations of cells were selected rather than single cell clones to minimize selecting individual clones that have phenotypically drifted from the parental 4T1 cells because of the lentiviral transduction. Five and 6 separate cell populations were generated for both PAR-1 and PAR-2 silenced 4T1 cells, respectively. Knockdown efficiency was determined by real time polymerase chain reaction (PCR) analysis. The cell populations with the highest silencing efficiency were used for the experiments.

Real time PCR.

RNA was isolated from cells using the RNeasy Plus kit (QIAGEN). mRNA was reverse transcribed using the First Strand cDNA Synthesis kit with Oligo-dT primers (Fermentas). Exon-spanning gene specific primers (Table 3.1) were synthesized by Integrated DNA Technologies. Real time PCR was performed on a Mastercycler Gradient (Eppendorf) using the Maxima SYBR Green qPCR Master Mix (Fermentas). Relative mRNA levels were quantified using the ΔΔCt method normalized to hypoxanthine-guanine phosphoribosyl transferase (HPRT)[36].

uPA and PAI-1 enzyme-linked immunosorbent assay.

Cells were grown in 12-well plates to form confluent monolayers. Cells were then starved overnight in SFM (Gibco). After starvation, new SFM containing coagulation factors were added to the wells. Conditioned media was collected, centrifuged at 5,000 x *g* for 5

Table 3.1: Real Time PCR Primers

PRIMER	SEQUENCE
Mouse TF fwd	5'-TCA AGC ACG GGA AAG AAA AC-3'
Mouse TF rev	5'-CAA AAT AGC CCA GGA AGC AG-3'
Mouse PAR-1 fwd	5'-CAG CCA GAA TCA GAG AGG ACA GA-3'
Mouse PAR-1 rev	5'-CCA GCA GGA CGC TTT CAT TT-3'
Mouse PAR-2 fwd	5'-AGC CGG ACC GAG AAC CTT-3'
Mouse PAR-2 rev	5'-GGA ACC CCT TTC CCA GTG ATT-3'
Mouse uPA fwd	5'-TTA CTG CAG GAA CCC TGA CAA CCA-3'
Mouse uPA rev	5'-TGC TAA GAG AGC AGT CAT GCA CCA-3'
Mouse PAI-1 fwd	5'-TTC AGT GGC CAA TGG AAG ACT CCT-3'
Mouse PAI-1 rev	5'-AGG GCA GTT CCA CAA CGT CAT ACT-3'
Mouse HPRT fwd	5'-CTG GTG AAA AGG ACC TCT CG-3'
Mouse HPRT rev	5'-TGA AGT ACT CAT TAT AGT CAA GGG CA-3'

minutes at 4°C to remove cellular debris, and frozen at -20°C. To obtain whole cell lysates, lysis buffer (10nM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], 1.5mM $MgCl_2$, 10mM KCl, 0.05% NP-40 [nonyl phenoxypolyethoxylethanol], pH 7.9) containing complete protease inhibitor cocktail was added to the cells. Cells were then scraped, sonicated, and centrifuged at 20,000 x *g* for 10 minutes at 4°C to pellet the cellular debris. The supernatant was then removed and stored at -20°C. uPA and PAI-1 enzyme-linked immunosorbent assays (ELISAs) were performed according to the manufacturer's protocol (Molecular Innovations).

uPA immunofluorescence.

Cells were grown on chamber slides (BD Biosciences), starved, fixed, and permeablized using BD Cytofix/Cytoperm (BD Biosciences). The cells were then incubated with fluorescein isothiocyanate (green) conjugated uPA antibody (Molecular Innovations) and Alexa Fluor-555 (red) conjugated Golgi marker GM130 antibody (BD Biosciences) for 1 hour at 4°C in the dark. Nuclei were counterstained with DAPI (blue). Slides were washed then mounted with HyrdoMount mounting medium (National Diagnostics).

Immunoblotting.

Cells were grown to confluence, starved overnight, and treated with either 125nM FXa, 20nM thrombin, or 200nM PMA. Cells were washed with cold phosphate-buffered saline and lysed in lysis buffer (Cell Signaling Technologies) containing 1mM dithiothreitol and phosphatase inhibitor cocktail. Samples were then sonicated and cleared of debris by centrifugation at 20,000 x *g* for 10 minutes at 4°C. Protein concentration was determined by

Bio-Rad protein assay and 50μg of lysate was combined with Laemmeli sample buffer, boiled, and the proteins were separated on a Novex Tris-Glycine 4% to 12% gradient gel (Invitrogen). Protein was transferred to polyvinylidene fluoride (PVDF) membranes (Millipore) and probed with an antibody against phosphorylated PKCμ at a 1:250 dilution (Cell Signaling Technologies) and an IRDye 800CW conjugated secondary antibody (Rockland) at a 1:10,000 dilution. The membranes were stripped in Restore Stripping Buffer (Thermo Scientific), blocked, and incubated with an antibody against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) at a 1:5,000 dilution (Santa Cruz Biotechnology) and an Alexa Fluor 680 conjugated secondary antibody (Invitrogen) at a 1:10,000 dilution. Blots were visualized and quantified using the Odyssey Infrared Imaging System (Licor).

Statistical analysis.

All statistical analyses were performed using GraphPad Prism 4 for Mac (GraphPad Software). All data are presented as means ± standard error of the mean (SEM). One-way analysis of variance with a Bonferroni posthoc analysis was performed when indicated. For 2 group comparisons, a 2-tailed Student *t* test was used. $P \leq .05$ was considered statistically significant.

RESULTS

PAR-1 and PAR-2 mediate crosstalk between coagulation and fibrinolysis.

Unstimulated 4T1 and 67NR cells expressed basal levels of TF, PAR-1, uPA, and PAI-1 mRNAs. Importantly, 4T1 cells expressed PAR-2 whereas the 67NR cells lacked detectable PAR-2 mRNA by real time PCR analysis (Figure 3.1-A and 3.1-B). It was

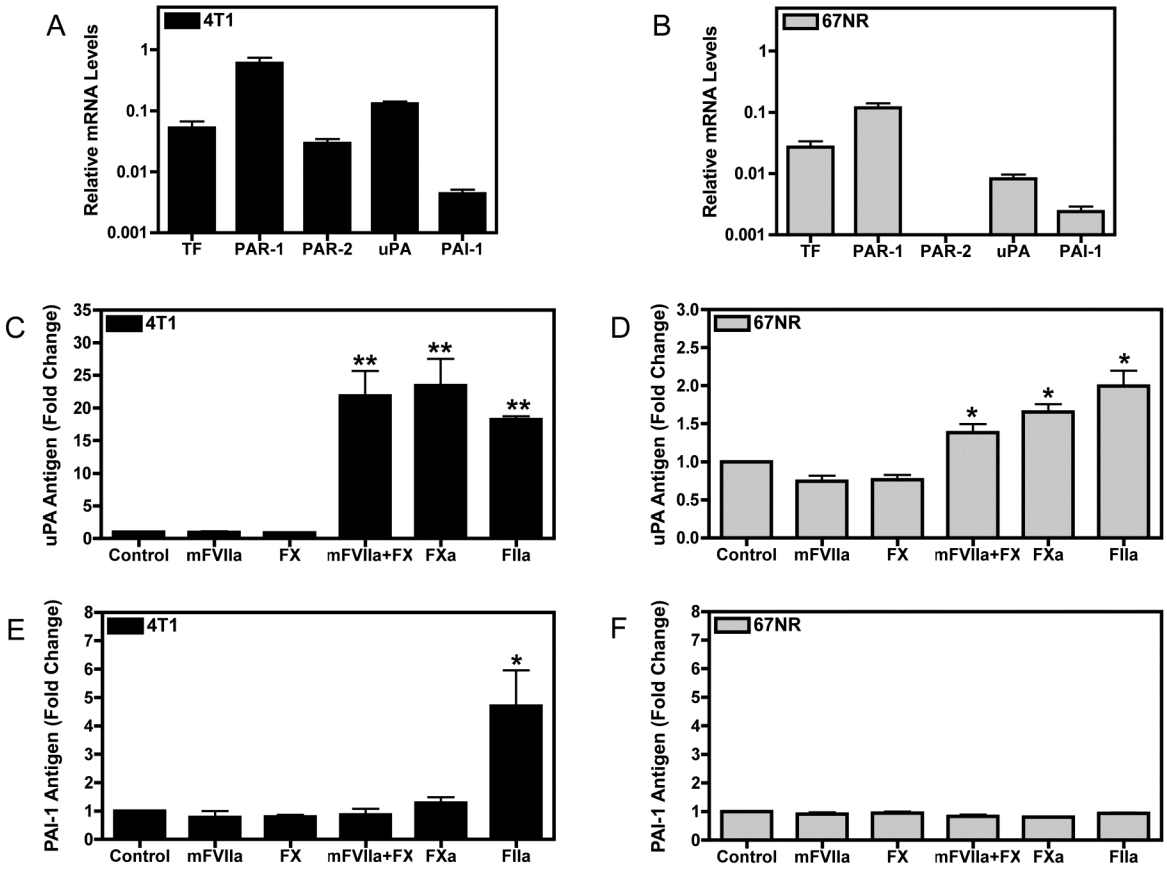

Figure 3.1. Coagulation proteases increase uPA and PAI-1 expression in the culture supernatant of 4T1 and 67NR breast cancer cell lines. (A-B) Real-time PCR analysis of TF, PAR-1, PAR-2, uPA, and PAI-1 mRNA expression in 4T1 cells (A) and 67NR cells (B). Cells were grown to confluence and starved overnight. Results are shown as mean ± SEM of at least three independent experiments. (C-E) Serum starved confluent cell monolayers were incubated 24 hours with the following coagulation factors: mFVIIa (10nM), FX (130nM), mFVIIa (10nM) and FX (130nM), FXa (125nM), or thrombin (FIIa; 20nM). Levels of uPA in treated 4T1 cells (C) and 67NR cells (D) were determined by ELISA. The amount of PAI-1 released from the treated 4T1 (E) and 67NR (F) cell lines was measured by a PAI-1 ELISA. Results are shown as mean ± SEM of at least five independent experiments. *P≤0.05 and **P≤0.001 (control versus protease treated).

determined using a one-stage clotting assay that 4T1 cells exhibited approximately 3-fold higher levels of TF activity than 67NR cells (data not shown). 4T1 cells were used for mFVIIa, FXa, and thrombin dose-titration experiments. mFVIIa did not increase the levels of either uPA or PAI-1 in the culture supernatant at 24 hours (Supplemental Figure S3.1-A and S3.1-B). FXa produced a dose-dependent increase of uPA but not PAI-1 (Supplemental Figure S3.1-C and S3.1-D). Treatment of the cells with thrombin led to dose-dependent increases of both uPA and PAI-1 in the cell culture supernatant (Supplemental Figure 3.1-E and S3.1-F).

Stimulation of 4T1 and 67NR cells with either mFVIIa or zymogen FX alone did not increase uPA levels in the culture supernatant (Figure 3.1C and 3.1-D). However, incubation of the 4T1 cells with both mFVIIa and FX resulted in high levels of uPA, whereas 67NR cells exhibited a more modest increase in uPA in response to the combination of mFVIIa and FX (Figure 3.1C and 3.1-D). The level of uPA generated when cells were incubated with both mFVIIa and FX was similar to the level observed with FXa alone (Figure 3.1C and 3.1-D), demonstrating that mFVIIa is enzymatically active. Similarly, thrombin increased uPA levels in the culture supernatant of both cell lines (Figure 3.1C and 3.1-D). Interestingly, thrombin was the only protease capable of increasing PAI-1 levels in the culture supernatant of 4T1 cells (Figure 3.1-E). Importantly, incubation of the 67NR cells with any of the proteases failed to increase PAI-1 levels in the cell culture supernatant at 24 hours (Figure 3.1-F). Similar results were obtained by adding mFVIIa, FX, a combination of mFVIIa and FX, FXa, or thrombin to 2 additional cell lines: the PAN02 pancreatic cancer cell line and the 168FARN breast cancer cell line (data not shown).

Absence of PAR-2 is associated with a loss of thrombin induced PAI-1 expression.

Our data demonstrated that cells lacking PAR-2 did not express PAI-1 in response to thrombin stimulation. Therefore, we hypothesized that PAR-2 is required for the induction of PAI-1. To test this hypothesis, we silenced PAR-2 expression in 4T1 cells to determine its role in uPA and PAI-1 expression. We also silenced PAR-1 expression in 4T1 cells. The different stably transduced shRNA cell populations are referred to as $4T1^{GFP}$, $4T1^{\Delta PAR\text{-}1}$, and $4T1^{\Delta PAR\text{-}2}$. PAR-1 and PAR-2 mRNA levels were knocked down in the $4T1^{\Delta PAR\text{-}1}$ and $4T1^{\Delta PAR\text{-}2}$ by 95% and 80%, respectively (Figure 3.2-A and 3.2-B). Silencing PAR-1 attenuated the FXa or thrombin-dependent increase of uPA protein (Figure 3.2-C). In contrast, levels of uPA in the culture supernatant of $4T1^{\Delta PAR\text{-}2}$ cells in response to FXa or thrombin did not significantly differ from those observed in the $4T1^{GFP}$ control cells (Figure 3.2-C). As expected, silencing PAR-1 diminished the thrombin-dependent increase of PAI-1. Importantly, silencing PAR-2 expression also drastically reduced PAI-1 induction in response to thrombin treatment (Figure 3.2-D), supporting our hypothesis that PAR-2 is required for inducible PAI-1 expression.

Furthermore, we used Gene Sifter Analysis Edition, Version 3.4 (Geospiza) to mine in vivo gene array data from laser capture microdissected cells from orthotopic 67NR and 4T1 primary tumors. The original data were submitted to the Gene Submission Omnibus database by Lou et al (GSE 11259)[37]. We found that 4T1 cells expressed significantly more PAR-2 (33.54-fold) and PAI-1 (9.56-fold) than 67NR cells in vivo. This suggests that the differential PAR-2 expression between the 67NR and 4T1 cells is not a consequence of in vitro culturing conditions and that PAR-2 may indeed be needed for inducible PAI-1 expression in vivo. Taken together, our data indicate that PAR-1 is required for FXa and thrombin mediated

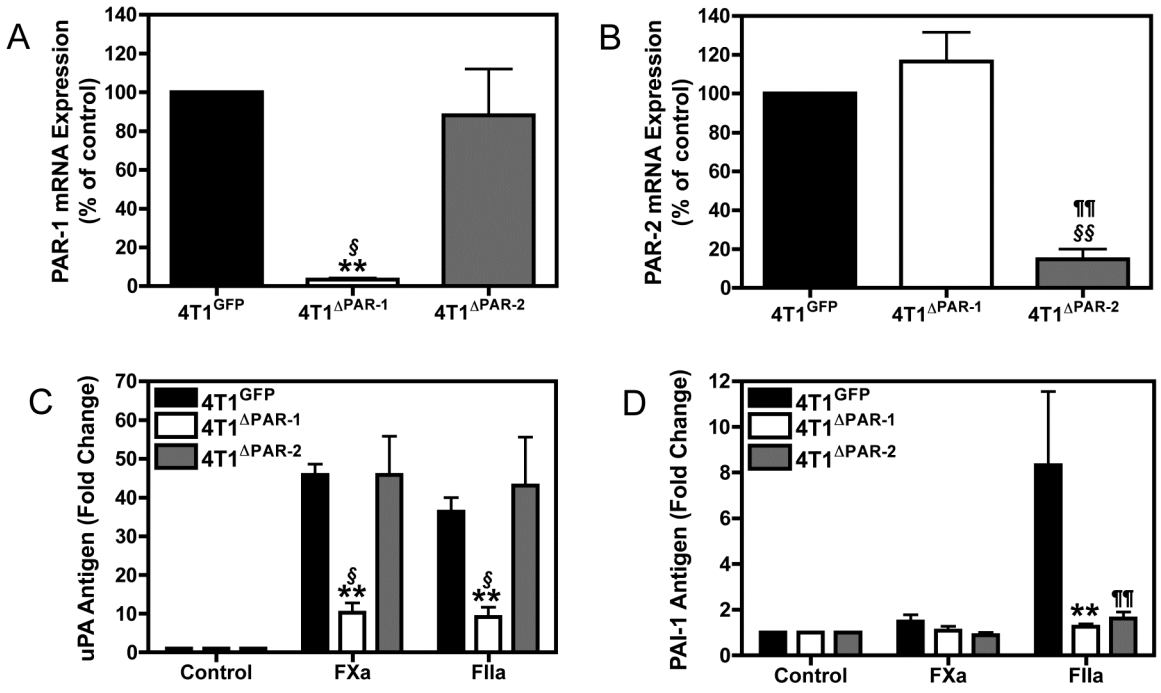

Figure 3.2. Silencing PAR-1 and PAR-2 in 4T1 cells. (A-B) Real-time PCR analysis of PAR-1 (A) and PAR-2 (B) mRNA expression in $4T1^{\Delta PAR-1}$ and $4T1^{\Delta PAR-2}$ cells expressed as a percentage of the $4T1^{GFP}$ control. PAR-1 and PAR-2 levels were normalized to HPRT mRNA. uPA protein (C) and PAI-1 protein (D) were measured by ELISA after 24 hour incubation with FXa (125nM) or thrombin (20nM). Results are shown as mean ± SEM of three independent experiments. *P≤0.05 and **P≤0.001 ($4T1^{GFP}$ versus $4T1^{\Delta PAR-1}$), ¶P≤0.05 and ¶¶P≤0.001 ($4T1^{GFP}$ versus $4T1^{\Delta PAR-2}$), §P≤0.05 and §§P≤0.001 ($4T1^{\Delta PAR-1}$ versus $4T1^{\Delta PAR-2}$).

increases in uPA whereas both PAR-1 and PAR-2 are required for thrombin-dependent increases in PAI-1.

Transcriptional and posttranslational regulation of PAI-1 and uPA.

To understand the mechanism by which PAR activation regulates uPA and PAI-1, we examined the kinetics of uPA and PAI-1 mRNA and protein expression in 4T1 cells treated with FXa or thrombin. uPA mRNA levels remained unchanged in 4T1 cells stimulated with either FXa or thrombin (Figure 3.3-A). This is in stark contrast to uPA protein, which was rapidly increased in the culture supernatant in response to a 1-hour treatment with FXa or thrombin, respectively (Figure 3.3-B). Further increases in uPA were observed throughout the 24-hour period. We also observed uPA release in response to a 1-hour incubation with FXa or thrombin in the PAN02 and 168FARN cell lines (data not shown).

Incubation of 4T1 cells with FXa did not significantly increase PAI-1 mRNA or protein levels (Figure 3.3-C and 3.3-D). In contrast, thrombin treatment of 4T1 cells increased PAI-1 mRNA levels, reaching a maximum induction of 2.2-fold before returning to baseline by 24 hours (Figure 3.3-C). An accumulation of PAI-1 protein in the culture supernatant was observed starting at 6 hours in thrombin treated 4T1 cells (Figure 3.3-D).

4T1 cells contain a store of intracellular uPA associated with the Golgi.

Analysis of uPA by direct immunofluorescence in SFM treated 4T1 cells under basal conditions revealed that the protease was stored in the perinuclear region of the cell (Figure 3.4-A). This uPA staining colocalized with the Golgi marker GM130, suggesting that intracellular uPA was associated with this secretory organelle. After treating the 4T1 cells for

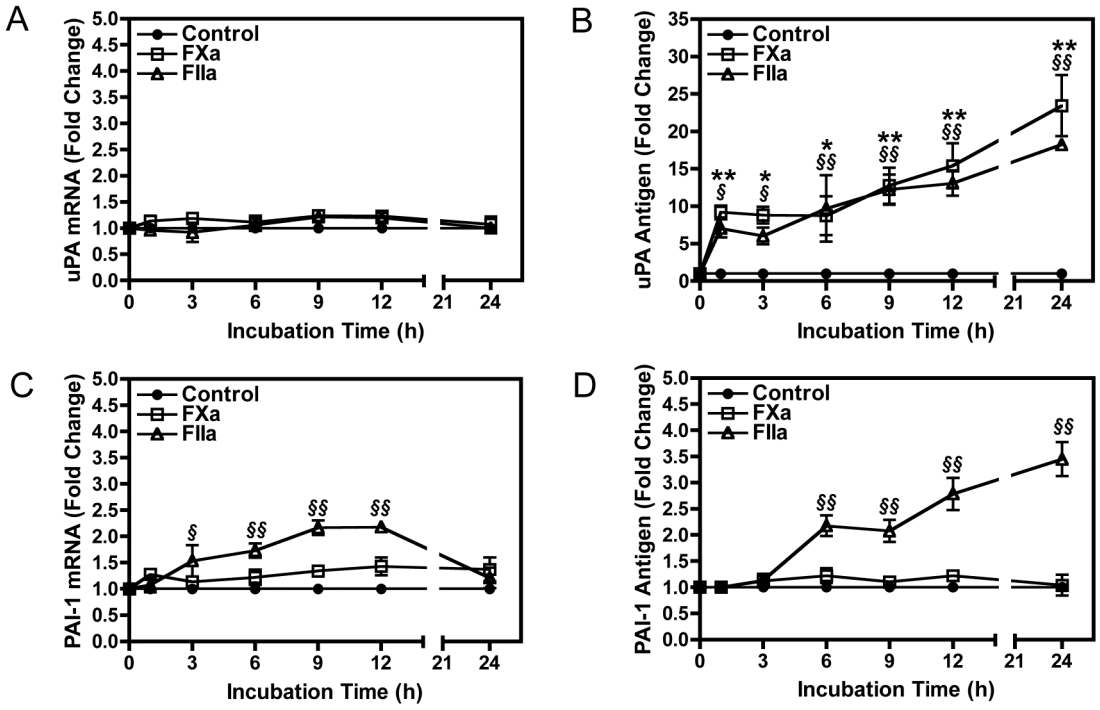

Figure 3.3. Time-course of uPA and PAI-1 mRNA and protein expression in 4T1 cells stimulated with FXa or thrombin. Levels of uPA mRNA (A) and protein (B) were determined by real-time PCR and ELISA, respectively. PAI-1 mRNA (C) and protein (D) induction were also measured using real-time PCR and ELISA. uPA and PAI-1 mRNA levels were normalized to HPRT mRNA. Results are shown as mean ± SEM of at least three independent experiments. *P≤0.05 and **P≤0.001 (control versus FXa treated). §P≤0.05 and §§P≤0.001 (control versus thrombin treated).

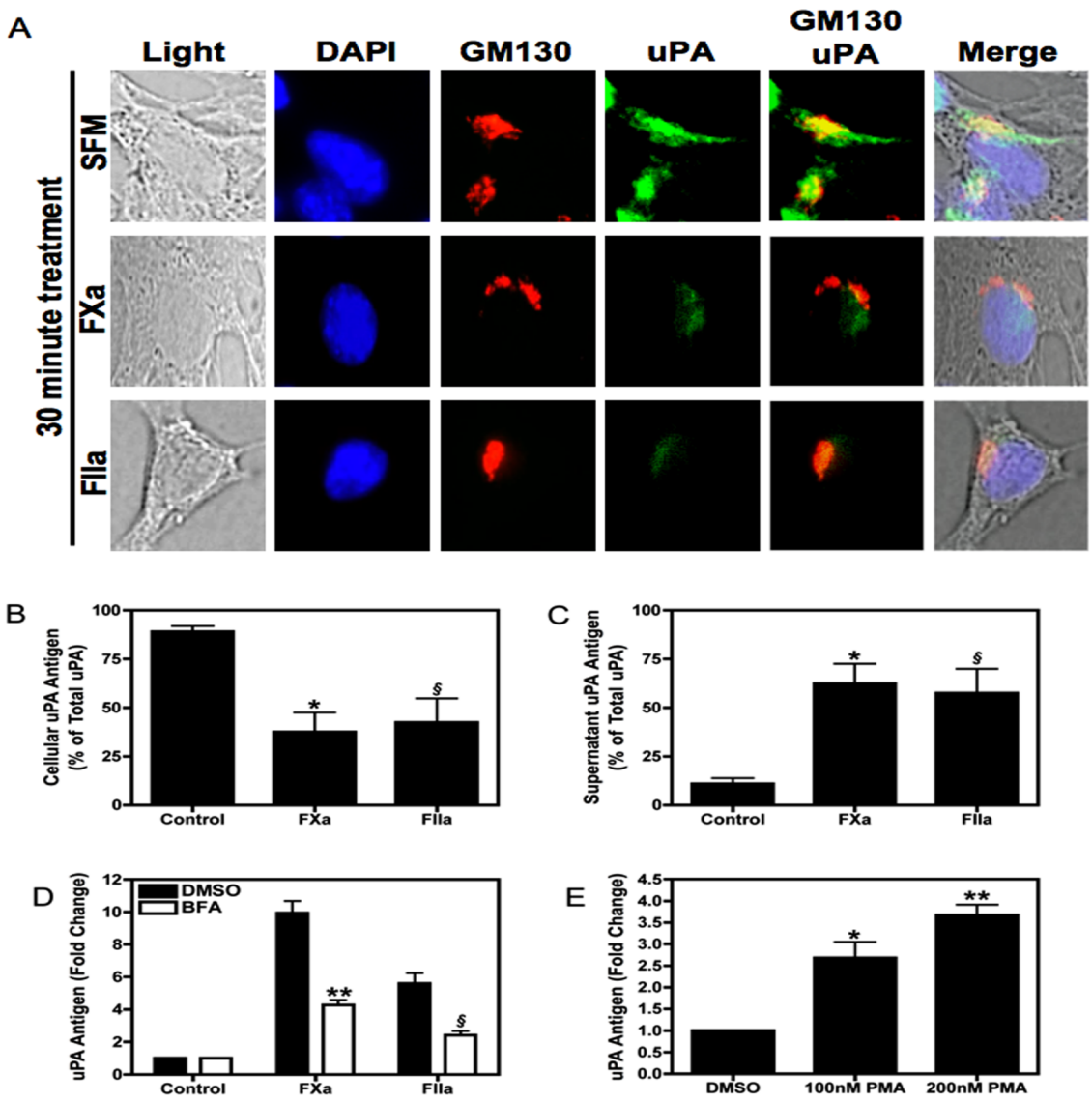

Figure 3.4. Stimulation of 4T1 cells with FXa or thrombin induces uPA secretion. (A) Intracellular uPA staining. Sub-confluent 4T1 cells were starved overnight then incubated with either SFM, FXa, or thrombin for 30 minutes. The cells were then fixed and permeablized on glass chamber slides. Cells were incubated with both uPA-FITC (green) and GM130-Alexa Fluor555 (red) antibodies, then counter-stained with DAPI (blue). Slides were viewed on an Olympus BX51WI fluorescence microscope fitted with an Olympus DP70 cooled digital color camera. Total magnification is 400X (10X ocular; 40X objective). DP Controller version 2.2.1.227 software was used for image acquisition. GraphicConverter X V5.4 was used to compile images. (B-C) Serum starved 4T1 cells were incubated with FXa (125nM) or thrombin (20nM) for 1 hour. Cellular (B) and culture supernatant (C) uPA expressed as percentage of total uPA in 4T1 cells. Results are shown as mean ± SEM of three independent experiments. *P≤0.05 (control versus FXa treated) and §P≤0.05 (control versus thrombin treated). (D) Cells were treated with BFA (10μg/mL) or vehicle control then stimulated with FXa (125nM) or thrombin (20nM) for 1 hr. uPA was quantified in cell culture supernatant. **P≤0.05 (control versus FXa treated) and §P≤0.05 (control versus thrombin treated). (E) uPA levels in the culture supernatant of 4T1 cells treated with 100nM or 200nM PMA for 1 hour. *P≤0.05 and **P≤0.001 (control versus PMA treated). Results are shown as mean ± SEM of three independent experiments.

30 minutes with either FXa or thrombin, we observed a marked decrease in the intensity of uPA staining (Figure 3.4-A). Next, we measured levels of uPA in whole cell lysates and culture supernatants of 4T1 cells with or without FXa or thrombin treatment. After a 1-hour incubation with FXa or thrombin, cellular uPA levels diminished whereas levels in the culture supernatant increased (Figure 3.4-B and 3.4-C).

Next, we used BFA to inhibit budding of newly formed secretory vesicles from the trans-Golgi network. FXa or thrombin mediated release of uPA was decreased by approximately 50% when cells were pre-treated with BFA (Figure 3.4-D). In addition, we directly activated the secretory pathway by incubating 4T1 cells with PMA. Exposure of 4T1 cells to PMA for 1 hour induced uPA release in a dose-dependent manner (Figure 3.4-E).

FXa and thrombin activate the secretory signaling pathway in 4T1 cells.

Protein kinase C-μ (PKCμ) is an intracellular signaling protein involved in secretion from the Golgi. Importantly, we detected phosphorylated PKCμ in 4T1 cells treated with FXa, thrombin, or PMA for 5, 10, or 15 minutes (Figure 3.5-A), which suggests that PKCμ is involved in uPA release. Our previous results demonstrate that activation of PAR-1 results in the rapid release of uPA in 4T1 cells. Therefore, we hypothesized that cleavage of PAR-1 induces PKCμ phosphorylation. $4T1^{GFP}$, $4T1^{\Delta PAR\text{-}1}$, and $4T1^{\Delta PAR\text{-}2}$ cells were treated with SFM, FXa, thrombin, or PMA for 5 minutes, and the cell lysates were used to detect phosphorylation of SFM, FXa, thrombin, or PMA for 5 minutes, and the cell lysates were used to detect phosphorylation of PKCμ. $4T1^{\Delta PAR\text{-}1}$ cells exhibited significantly decreased levels of PKCμ. $4T1^{\Delta PAR\text{-}1}$ cells exhibited significantly decreased levels of phosphorylated PKCμ when treated with either FXa or thrombin in comparison to the $4T1^{GFP}$

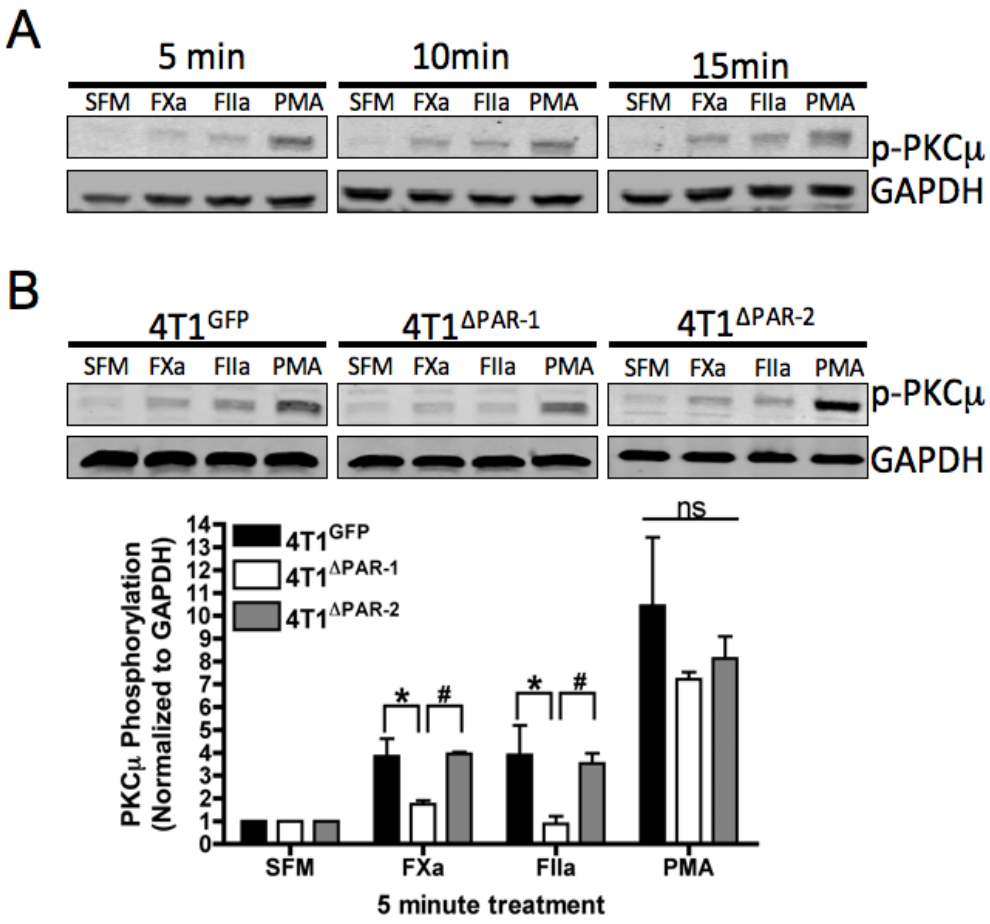

Figure 3.5. Coagulation proteases activate the secretory pathway in the 4T1 cell line. (A) 4T1 cells were treated for the indicated amount of time with SFM, FXa, thrombin, or 200nM PMA. Blots were probed with antibodies for p-PKCμ and GAPDH. Data shown is representative of three independent experiments. (B) $4T1^{GFP}$, $4T1^{\Delta PAR-1}$, and $4T1^{\Delta PAR-2}$ cells were treated with SFM, FXa, thrombin, or PMA for 5 minutes. Blots were probed with antibodies for p-PKCμ and GAPDH. Phosphorylated PKCμ was quantified by dividing the background-corrected p-PKCμ signal intensity by the background corrected GAPDH signal intensity. The blot shown is representative of at least three independent experiments. Quantification results are shown as mean ± SEM of at least three independent experiments. *P≤0.05 ($4T1^{GFP}$ versus $4T1^{\Delta PAR-1}$) and $^{\#}$P≤0.05 ($4T1^{\Delta PAR-1}$ versus $4T1^{\Delta PAR-2}$).

and 4T1$^{\Delta PAR\text{-}2}$ (Figure 3.5-B), thereby supporting our hypothesis that cleavage of PAR-1 activates the secretory pathway.

DISCUSSION

Our results show that FXa or thrombin mediated activation of PAR-1 induces a rapid release of uPA in murine mammary adenocarcinoma cells (Figure 3.6). In contrast to previous reports using human cells lines, we did not find evidence for the induction of uPA mRNA expression in the murine tumor lines used in this study[31,32,38]. Cellular uPA colocalized with the Golgi. In addition, inhibiting secretion with BFA decreased uPA protein release. BFA collapses the trans-Golgi network, thereby preventing trafficking of immature and newly synthesized secretory vesicles[39]. The portion of uPA not inhibited by BFA treatment presumably represents uPA that is already within mature secretory vesicles. Furthermore, uPA was released by PMA activation of the secretory pathway. Activated PKCμ translocates to the Golgi apparatus where it is involved in vesicular trafficking from the trans-Golgi network to the plasma membrane[40]. Phosphorylation of the 2 activation loops of PKCμ resulting from FXa or thrombin treatment is indicative of activation of this secretory pathway. Taken together, these data demonstrate a novel mechanism of uPA regulation downstream of cellular activation by FXa or thrombin.

Both PAR-1 and PAR-2 are required for PAI-1 mRNA and protein expression because silencing of either receptor in 4T1 cells abolished the increase of PAI-1 in the culture supernatant. This result is consistent with the absence of inducible PAI-1 expression in breast tumor lines lacking PAR-2, such as 67NR cells. This codependency suggests that PAR-1 and PAR-2 are complexed together and that cleaved PAR-1 transactivates PAR-2 to

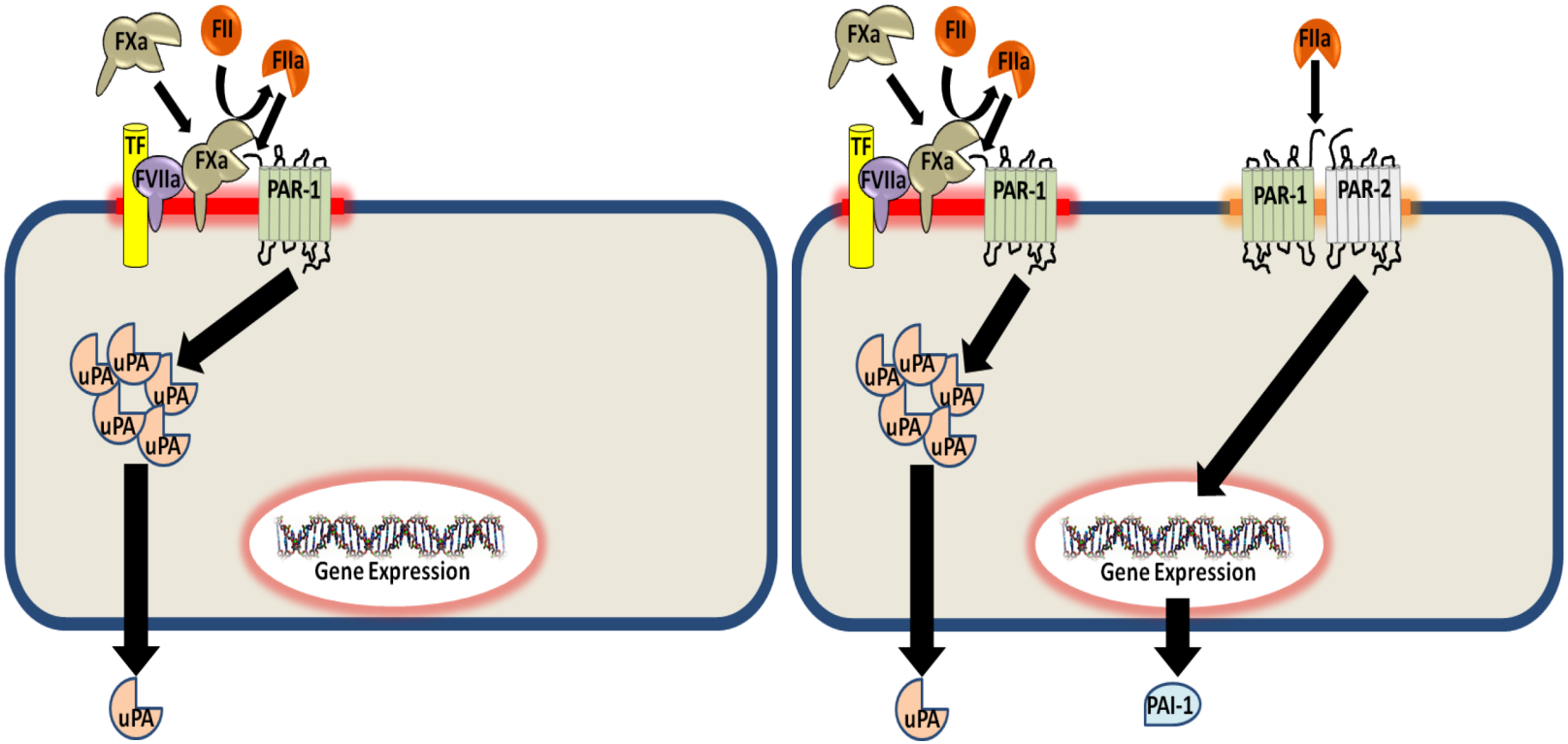

Figure 3.6. Proposed model of how coagulation protease activation of PAR-1 and PAR-2 regulates uPA and PAI-1 expression in mouse breast cancer cell lines. Both PAR-1 and PAR-2 can exist as individual receptors or in a complex. Cells, such as the 67NR cell line (left panel), can express PAR-1 only whereas 4T1 cells express PAR-1 and a PAR-1/PAR-2 complex (right panel). The configuration of PAR-1 and PAR-2 determines the expression of uPA and PAI-1 in response to different coagulation proteases

induce PAI-1 expression. Interestingly, thrombin, but not FXa, activated the PAR-1/PAR-2 complex. These data are consistent with the thrombin-dependent activation of a PAR-1/PAR-2 complex previously described by O'Brien *et al*[21]. Thrombin activated PAR-1 and transactivated PAR-2 have been shown to use separate intracellular signaling pathways in an endothelial cell model of sepsis using pharmacological inhibitors and siRNA against PAR-1 or PAR-2[41]. This may explain the differential regulation of uPA secretion and PAI-1 mRNA expression by PAR-1 and a PAR-1/PAR-2 complex, respectively, in tumor cells.

Our model suggests that there is differential signaling in tumor cells containing either PAR-1 alone or both PAR-1 and PAR-2 (Figure 3.6). In cells expressing only PAR-1, FXa or thrombin activate the receptor resulting in uPA secretion. Thrombin activates PAR-1 in a membrane-independent manner whereas FXa is anchored to the membrane. This membrane tethering may be achieved when FXa is complexed with TF-FVIIa or when FXa is bound to the membrane. In cells that express both PAR-1 and PAR-2, we found evidence of a PAR-1/PAR-2 complex. In these cells, we propose that PAR-1, the more abundantly expressed receptor, can exist as a lone receptor or as part of the PAR-1/PAR-2 complex, whereas all of the PAR-2 is sequestered into the complex. Importantly, unlike PAR-1 alone, the PAR-1/PAR-2 complex is activated by thrombin but not by FXa. This thrombin-dependent cleavage of PAR-1 transactivates PAR-2 resulting in increased PAI-1 expression. Previous studies have shown that receptors, such as TF and PARs, are often clustered together in specialized membrane microdomains, namely caveolae and lipid rafts, to enhance signaling[42]. Presumably, PAR-1 and the PAR-1/PAR-2 complex reside in different membrane domains explaining why only a trans-acting protease, such as thrombin, is capable of activating both sets of PARs on the cell surface.

We were unable to observe a TF-FVIIa-PAR-2 dependent regulation of either uPA or PAI-1 in the 4T1 cell line. In contrast, TF-FVIIa-PAR2 signaling has been described using the human breast cancer cell line MDA-MB-231[31,43]. Using Oncomine (Compendia Biosciences), we mined gene array data (GSE 2603) and found that the mRNA levels of TF and PAR-2 were significantly greater than that of PAR-1 in MDA-MB-231 tumor xenografts[44]. We found similar results by quantitative real-time PCR using cultured MDA-MB-231 cells (data not shown). TF-FVIIa-PAR-2 signaling was also observed in baby hamster kidney cells transfected with TF and human endothelial cells transfected with TF and PAR-2[45]. Taken together, this suggests that the levels of TF and PAR-2 expressed on the 4T1 cells are not high enough to support for TF-FVIIa-PAR-2 signaling.

PARs enable cells to detect, and therefore respond to proteases present in the local environment. Tumor metastasis and angiogenesis, whether lymphatic or hematogenous, requires a variety of matrix remodeling proteases, including matrix metalloproteinase-1 and plasmin[46,47]. These proteases, in addition to mast cell tryptase, and tissue kallikreins, are present within the breast tumor stroma and are known to activate PAR-1 or PAR-2[18,48,49]. Recently, it has been demonstrated that oncogenes increase TF, PAR-1, and PAR-2 expression[2]. We hypothesize that the local generation of FXa and thrombin on the surface of tumor cells, in addition to the aforementioned proteases present in the tumor stroma, may activate PAR-1 or PAR-2, leading to increased release/expression of uPA and PAI-1. Both uPA and PAI-1 have established roles in matrix degradation, tumor motility, and angiogenesis[22]. Our study explains how the coagulation system may use PAR-1 and PAR-2 to promote malignancy via increased generation of plasmin.

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AUTHORSHIP

Contribution.

T.A.M. designed the experiments, performed the experiments, interpreted the data, and wrote the manuscript; R.P. aided in experimental design, data interpretation, and critically read and edited the manuscript; K.L.R. provided invaluable technical assistance and critically read the manuscript; F.C.C. aided in data interpretation and critically read the manuscript; and N.M. designed the experiments, interpreted the data, edited the manuscript, and contributed to the overall design of the study.

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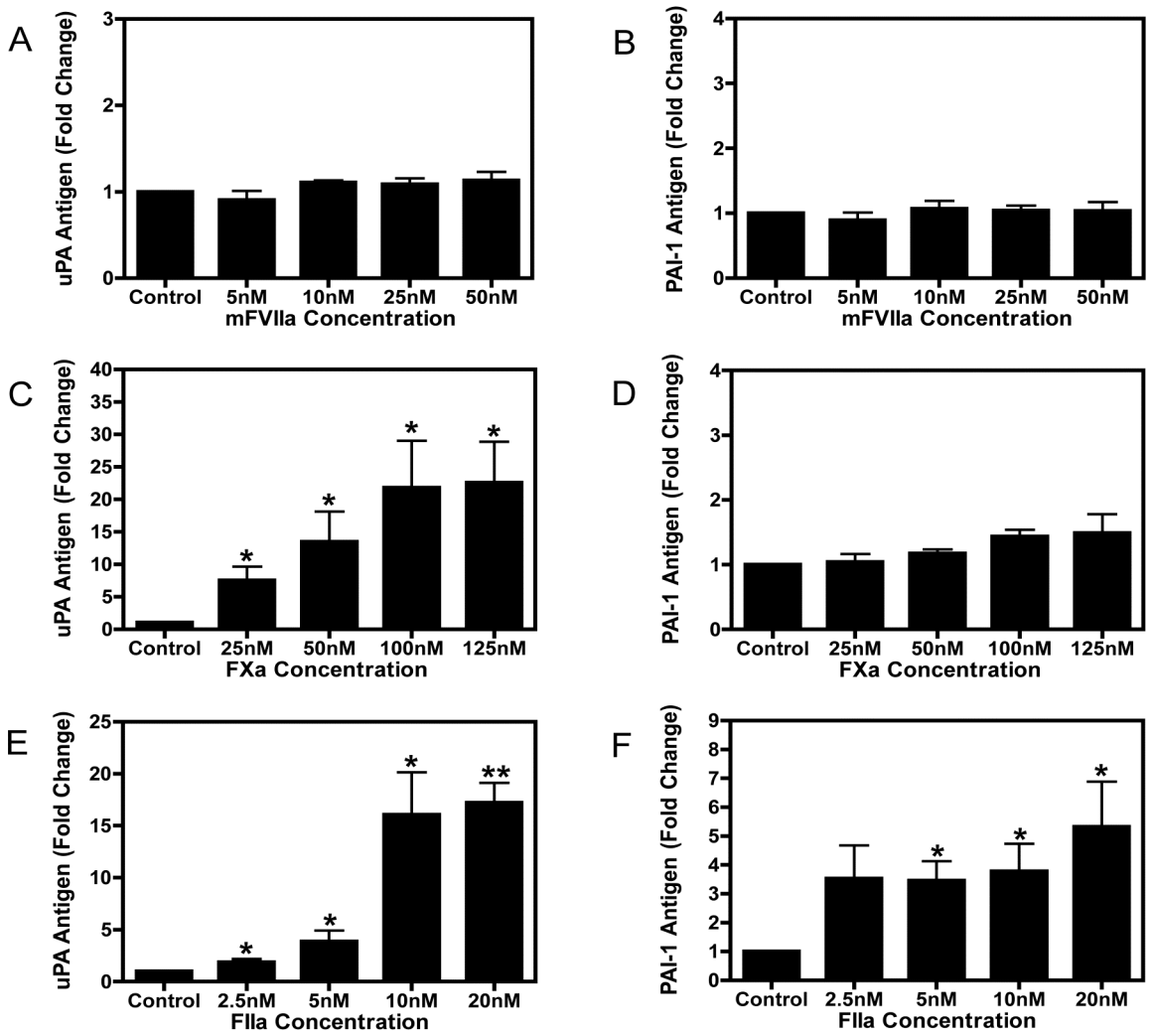

Supplemental Figure S3.1: mFVIIa, FXa, and thrombin dose titration in 4T1 cells. Serum starved confluent cell monolayers were incubated with the indicated concentrations of FXa and thrombin for 24 hours. Levels of uPA in cell culture supernatant of 4T1 cells treated with mFVIIa (A), FXa (C), and thrombin (E) were determined by ELISA. PAI-1 levels the in cell culture supernatant of 4T1 cells treated with mFVIIa (B), FXa (D), and thrombin (F) was measured by ELISA. Results are shown as mean ± SEM of at least five independent experiments. *P≤0.05 and **P≤0.001 (control versus protease treated).

CHAPTER IV:

Regulation of Thrombin-Induced Plasminogen Activator Inhibitor-1 in 4T1 Murine Breast Cancer Cells

This research is currently being submitted for publication in *Blood Coagulation and Fibrinolysis*.

McEachron TA, Church FC, Mackman N. Regulation of Thrombin-Induced Plasminogen Activator Inhibitor-1 in 4T1 Murine Breast Cancer Cells.

ABSTRACT

Protease-activated receptor-1 (PAR-1) and PAR-2 are overexpressed in cancer cells and activation of these receptors contributes to malignancy. We have recently shown that thrombin activates PAR-1, which induces transactivation of PAR-2, resulting in increased plasminogen activator inhibitor-1 (PAI-1) expression in 4T1 murine mammary adenocarcinoma cells. We analyzed the signal transduction pathways that regulate thrombin-induced PAI-1 expression. Thrombin stimulation activates the ERK1/2-ELK1-EGR1 pathway. Furthermore, inhibition of p42/p44 MAPK signaling reduced PAI-1 expression. These results begin to delineate the mechanism by which thrombin activates a PAR-1/PAR-2 complex to induce PAI-1 expression in the 4T1 murine breast cancer cell line.

INTRODUCTION

The G-protein coupled receptor (GPCR) family encompasses a large number of seven-pass transmembrane receptors. Included in this family is the sub-family of protease-activated receptors (PARs)[1]. As their name suggests, PARs are activated by a proteolytic cleavage in their extracellular N-terminus[2,3]. There are four PARs, PAR-1, -2, -3, and -4. Of interest, PAR-1 is activated by the coagulation proteases FXa and thrombin[3,4]. Other PAR-1 agonists include matrix metalloproteinase-1, plasmin, and activated protein C[5-8]. PAR-2 is activated by coagulation proteases FVIIa and FXa, and by trypsin, tryptase, and kallikriens[9-12]. Additionally, thrombin-activated PAR-1 transactivates PAR-2 in both human and mouse cells[13,14]. PAR-1 and PAR-2 activation has been reported to induce numerous intracellular signaling pathways, including the nuclear factor kappa B (NFκB) and p42/p44 mitogen activate protein kinase (MAPK) pathways[15]. In human breast cancer cells, PAR-dependent

signaling induces the expression genes that favor tumor progression, such as plasminogen activator inhibitor-1 (PAI-1)[16]. We recently demonstrated that PAI-1 is induced by PAR-1/PAR-2 transactivation[14].

PAI-1 is a serine protease inhibitor (SERPIN) that inhibits fibrinolysis by binding to the active sites of urokinase plasminogen activator (uPA) and tissue plasminogen activator (tPA)[17,18]. PAI-1 is a pleiotropic molecule that functions in hemostasis, angiogenesis, and vessel wall repair[19]. Additionally, PAI-1 is widely expressed in a variety of cell types and contributes to cancer pathology by enhancing tumor angiogenesis, survival, and invasion[20,21]. PAI-1 is positively regulated by numerous signaling pathways including the phosphoinositide 3-kinase (PI3K), p42/p44 MAPK, p38 MAPK, NFκB, and beta catenin (β-catenin) pathways, amongst others[22]. These pathways increase PAI-1 expression in a cell type dependent manner. In human breast cancer cells, the transcription factor ets-like gene-1 (ELK1) directly increases PAI-1 transcription in a p42/p44 MAPK-dependent manner[23]. Furthermore, in a murine liver cancer model, PAI-1 expression is driven by oncogenic *MET*[24]. Moreover, PAI-1 expression can be increased by soluble mediators, such as transforming growth factor beta (TGF-β), and by environmental conditions, such as hypoxia, both of which are stimuli in breast cancer[25,26]. In this study, we examined select intracellular signaling pathways and transcription factors that could regulate thrombin-induced PAI-1 expression in 4T1 murine mammary adenocarcinoma cells. We conclude that thrombin-induced PAI-1 expression is regulated by PAR-2-dependent activation of the ERK1/2-ELK1-EGR1 pathway.

MATERIALS AND METHODS

Reagents.

Purified human activated coagulation factor X (FXa) and α-thrombin (FIIa) were obtained from Haematologic Technologies Inc. Dimethyl sulfoxide (DMSO), dithiothreitol (DTT), penicillin/streptomycin, guanidine HCl, nonyl phenoxypolyethoxylethanol (NP-40), and tris(hydroxymethyl)aminomethane (Tris) were obtained from Sigma. 4-β-phorbol-12-myristate 13-acetate (PMA) was purchased from Cell Signaling Technologies. 1,4-Diamino-2,3-dicyano-1,4-*bis*(2-aminophenylthio)butadiene (U0126) and the PAR-2 agonist peptide 2f-LIGRLO-amide were purchased from Calbiochem. Complete protease inhibitor cocktail tablets and phosphatase inhibitor cocktail were obtained from Roche.

Cell culture.

4T1 cells were provided by Dr Fred Miller (Michigan Cancer Foundation). 4T1 cells were routinely cultured in minimal essential medium (MEM)-alpha (Gibco) supplemented with 10% fetal bovine serum (FBS; Omega Scientific), and 1% penicillin/streptomycin. Cells were grown to 100% confluence in 12-well tissue culture plates (Corning Inc.) then starved for 16 hours in serum-free media (SFM). Following serum starvation, fresh SFM containing FXa (125nM) or thrombin (20nM) was added to the wells. Complete culture media supplemented with 200nM PMA was used as a positive control (FBS+PMA). For inhibitor studies, U0126 (10μM) or DMSO (volume/volume), the vehicle control, was added to the cells 1 hour prior to agonist treatment.

Immunoblotting.

Following agonist treatment, cells were washed with ice-cold phosphate buffered saline and lysed in lysis buffer (Cell Signaling Technologies) supplemented with 1mM DTT and phosphatase inhibitor cocktail. Cells were scraped and transferred into 1.5mL microcentrifuge tubes and sonicated on ice for 30 seconds. The lysates were cleared of debris by centrifugation at 20,000 x g for 10 minutes at 4^{o}C. The Bio-Rad DC protein assay (Bio-Rad Laboratories) was used to determine the protein concentration in each sample. Protein lysates were combined with Laemmeli sample buffer and boiled at 100^{o}C for 5 minutes. The reduced lysates were separated on a Novex Tris-Glycine 4%-12% gradient polyacrylimide gel (Invitrogen). The proteins were transferred to Immobilon-FL PVDF membranes (Millipore). The membranes were probed with antibodies against phosphorylated extracellular signal-regulated kinase 1/2 (pERK1/2), total ERK1/2 (ERK1/2), phosphorylated ELK1 (pELK1), early growth response-1 (EGR1; Santa Cruz Biotechnology), or inhibitory kappa B alpha (IκBα; Cell Signaling Technologies). ELK1 and EGR1 blots were stripped in stripping buffer (6M guanidine HCl, 0.2% NP-40, 20mM Tris, 10mM dithiothreitol, pH 7.5) then probed with antibodies against total ELK1 or glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Santa Cruz Biotechnology), respectively. Alexa Fluor 680 and Alexa Fluor 800 conjugated secondary antibodies (Invitrogen) were used. The membranes were scanned and bands quantified using the Odyssey Infrared Imaging System (Licor Biosciences).

Real time PCR.

RNA was isolated from cells using the RNeasy Plus kit (Qiagen) following the manufacturer's protocol. mRNA was reverse transcribed using the First Strand cDNA Synthesis kit with Oligo-dT primers (Fermentas). EGR1 and hypoxanthine-guanine phosphoribosyltransferase (HPRT) primers were synthesized by Integrated DNA Technologies. Primer sequences are as follows: EGR1 forward 5'-AAC AAC CCT ATG AGC ACC TGA CCA-3'; EGR1 reverse 5'-AGT CGT TTG GCT GGG ATA ACT CGT-3'; HPRT forward 5'-CTG GTG AAA AGG ACC TCT CG-3'; HPRT reverse 5'-TGA AGT ACT CAT TAT AGT CAA GGG CA-3'. Real time PCR was performed on a Mastercycler Gradient (Eppendorf) using the Maxima SYBR Green qPCR Master Mix (Fermentas). The ΔΔCt method was used to calculate the relative mRNA levels[27]. EGR1 mRNA levels were normalized to the levels of HPRT mRNA.

PAI-1 enzyme-linked immunosorbent assay (ELISA).

Following 24-hour agonist treatment, cell culture supernatants were collected and cleared of debris by centrifugation at 5,000 x g for 5 minutes at 4^{o}C. A murine total antigen PAI-1 ELISA was used to quantify PAI-1 levels in the cell culture supernatants (Molecular Innovations).

Statistical analysis.

All statistical analyses were performed using GraphPad Prism 4 for Mac (GraphPad Software). All data are presented as means ± standard error of the mean (SEM). Two-tailed student *t* tests were used to determine statistical significance. Real time PCR data was analyzed using a two-way analysis of variance (ANOVA) with a Bonferroni post-hoc

analysis. P values ≤ 0.05 were considered statistically significant.

RESULTS

FXa and thrombin activate the p42/p44 MAPK signaling pathway in 4T1 cells.

The agonist incubation time points were chosen based on unpublished preliminary data obtained using the human breast cancer cell line MDA-MB-231. We found that FXa and thrombin activated the p42/p44 MAPK signaling pathway in 4T1 cells. Western blot analysis revealed that FXa and thrombin both significantly increased the phosphorylation of ERK1/2 (Figure 4.1-A). The p42/p44 MAPK pathway was further explored by examining downstream targets. The transcription factor ELK1 is phosphorylated by pERK1/2[28]. Stimulating 4T1 cells with FXa or thrombin for 5 minutes increased ELK1 phosphorylation (Figure 4.1-B). The NFκB pathway was also analyzed. 4T1 cells stimulated with the positive control, FBS+PMA, induced IκBα degradation, indicating activation of the NFκB pathway. However, neither FXa nor thrombin activated this signaling pathway in 4T1 cells (Figure 4.1-C).

Thrombin, but not FXa, induces EGR1 mRNA and protein expression in 4T1 cells.

Phosphorylated ELK1 (pELK1) has been shown to induce the expression of the transcription factor EGR1[29]. A time course experiment was performed to measure EGR1 mRNA levels by real time PCR. In response to thrombin stimulation, EGR1 mRNA peaked at 2 hours before returning to baseline (Figure 4.2-A). Interestingly, FXa did not increase EGR1 mRNA levels over the 6-hour time course. 2f-LIGRLO-amide, a PAR-2 specific agonist peptide, also

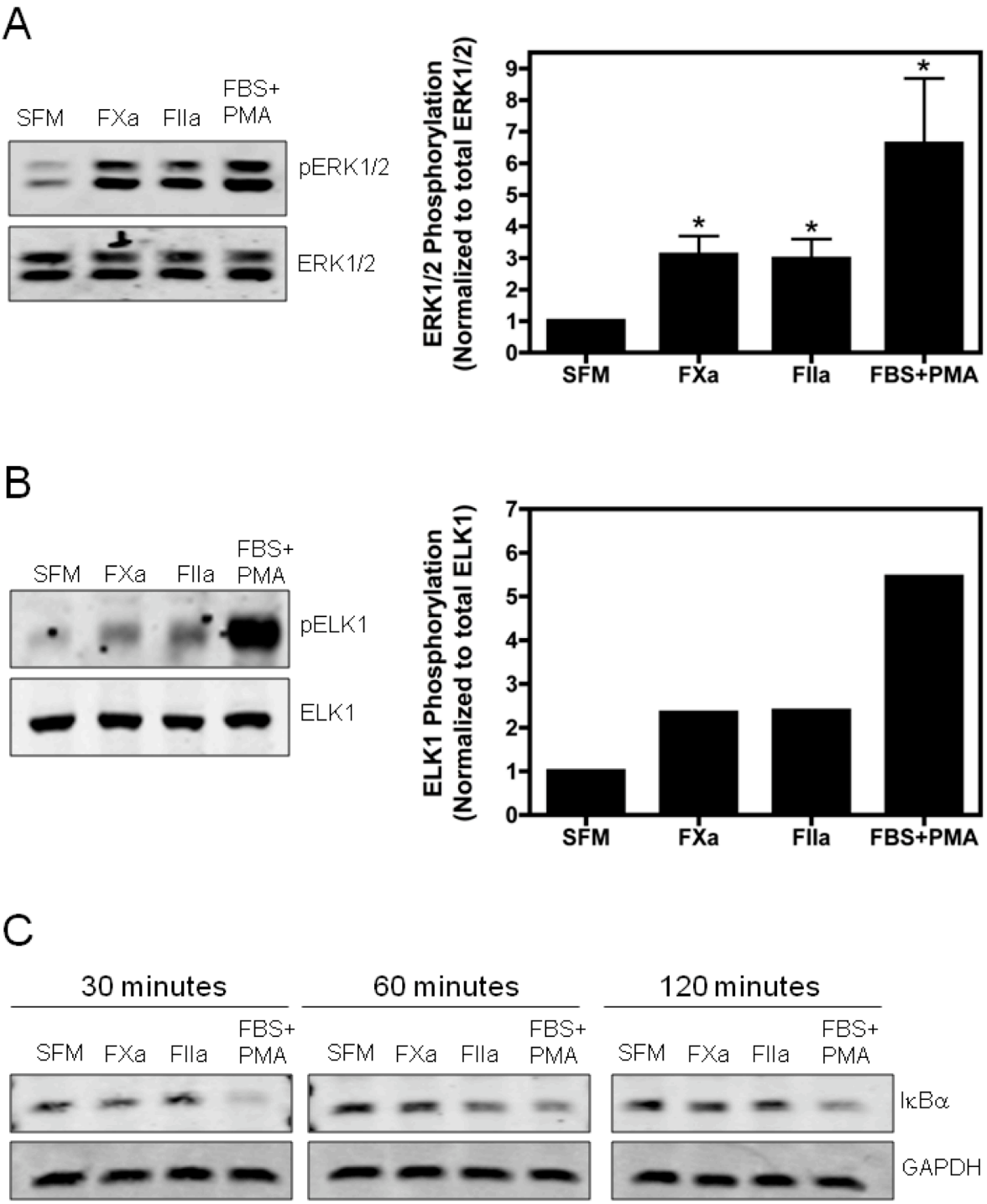

Figure 4.1. FXa and thrombin activate p42/p44 MAPK signaling and ELK1 in 4T1 cells. Confluent 4T1 cells were stimulated with 125nM FXa, 20nM thrombin (FIIa), or 10% FBS+200nM PMA (FBS+PMA) for 5 minutes and blotted for pERK1/2 and total ERK1/2 (A), pELK1 and total ELK1 (B), or IκBα and GAPDH (C). The pERK1/2 and pELK1 band intensities were quantified and normalized to the total ERK1/2 and total ELK1 band intensity, respectively. Results are shown as mean ± SEM of four (A) or three (C) independent experiments. *P≤0.05 (treated versus SFM). The pELK1 blot is representative of two independent experiments.

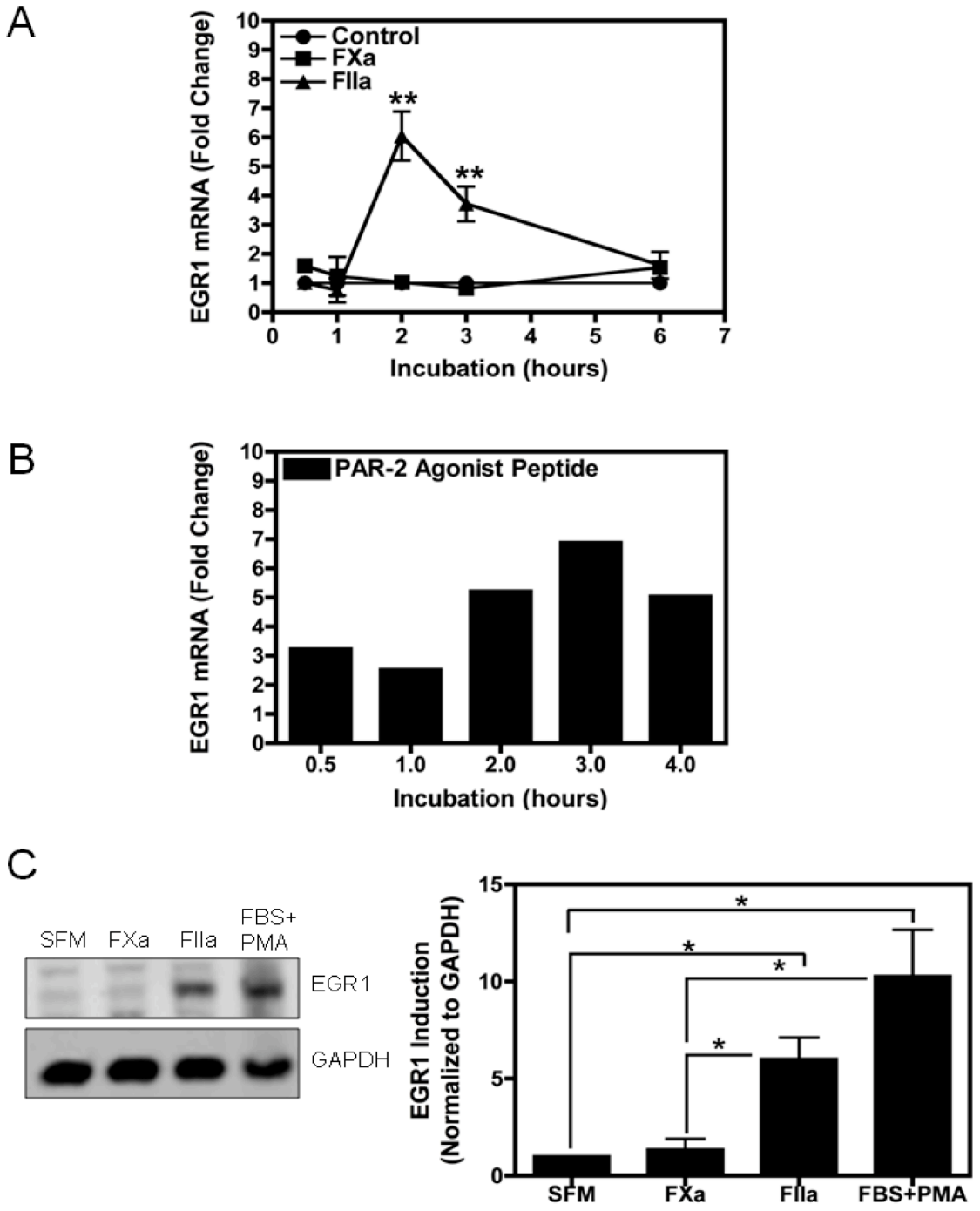

Figure 4.2. EGR1 is expressed in 4T1 cells in response to thrombin. Time course of EGR1 mRNA expression in 4T1 cells stimulated with 125nM FXa or 20nM thrombin (FIIa) (A), or 10μM PAR-2 agonist peptide (B). mRNA levels at the indicated time points were measured by real time PCR. Results are shown as mean ± SEM of three independent experiments (A). P-values were calculated using a two-way ANOVA with a Bonferroni post-hoc analysis. ** P≤0.01 (FIIa versus control). PAR-2 agonist peptide graph represents two independent experiments. (C) 4T1 cells were incubated with 125nM FXa, 20nM thrombin (FIIa), or 10% FBS+200nM PMA (FBS+PMA) for 3 hours and blotted for EGR1 and GAPDH (left). The pEGR1 band intensity was quantified and normalized to the GAPDH band intensity (right). Results are shown as mean ± SEM of three independent experiments. *P≤0.05.

increased EGR1 mRNA levels in 4T1 cells in a time-dependent manner (Figure 4.2-B). In this experiment, EGR1 mRNA levels were highest at 3 hours. EGR1 protein levels were also examined. Western blot analysis of 4T1 whole cell lysates showed that thrombin induced EGR1 protein expression while FXa had no effect (Figure 4.2-C).

Thrombin-induced PAI-1 expression in 4T1 cells requires the p42/p44 MAPK signaling pathway.

Previous studies show that p42/p44 MAPK signaling positively regulates PAI-1 expression in breast cancer cells and that the mouse PAI-1 promoter has at least one EGR1 binding site[30]. To demonstrate that the p42/p44 MAPK signaling pathway is necessary for thrombin-induced PAI-1 expression, the MEK inhibitor U0126 was used. Treating 4T1 cells with U0126 before stimulation with FXa or thrombin reduced the levels of pERK1/2 (Figure 4.3-A). U0126 attenuated the induction of pELK1 in response to FXa or thrombin (Figure 4.3-B). Thrombin-induced EGR1 protein expression was also decreased in 4T1 cells pre-treated with U0126 (Figure 4.3-C). Surprisingly, U0126 did not decrease ELK1 phosphorylation in 4T1 cells treated with FBS+PMA (Figure 4.3-B) but did decrease pERK1/2 and EGR1 protein levels in cells treated with the positive control (Figure 4.3-A and Figure 4.3-C). Inhibition of the p42/p44 MAPK signaling pathway also significantly reduced PAI-1 accumulation in the culture supernatant of 4T1 cells incubated with thrombin for 24 hours (Figure 4.3-D).

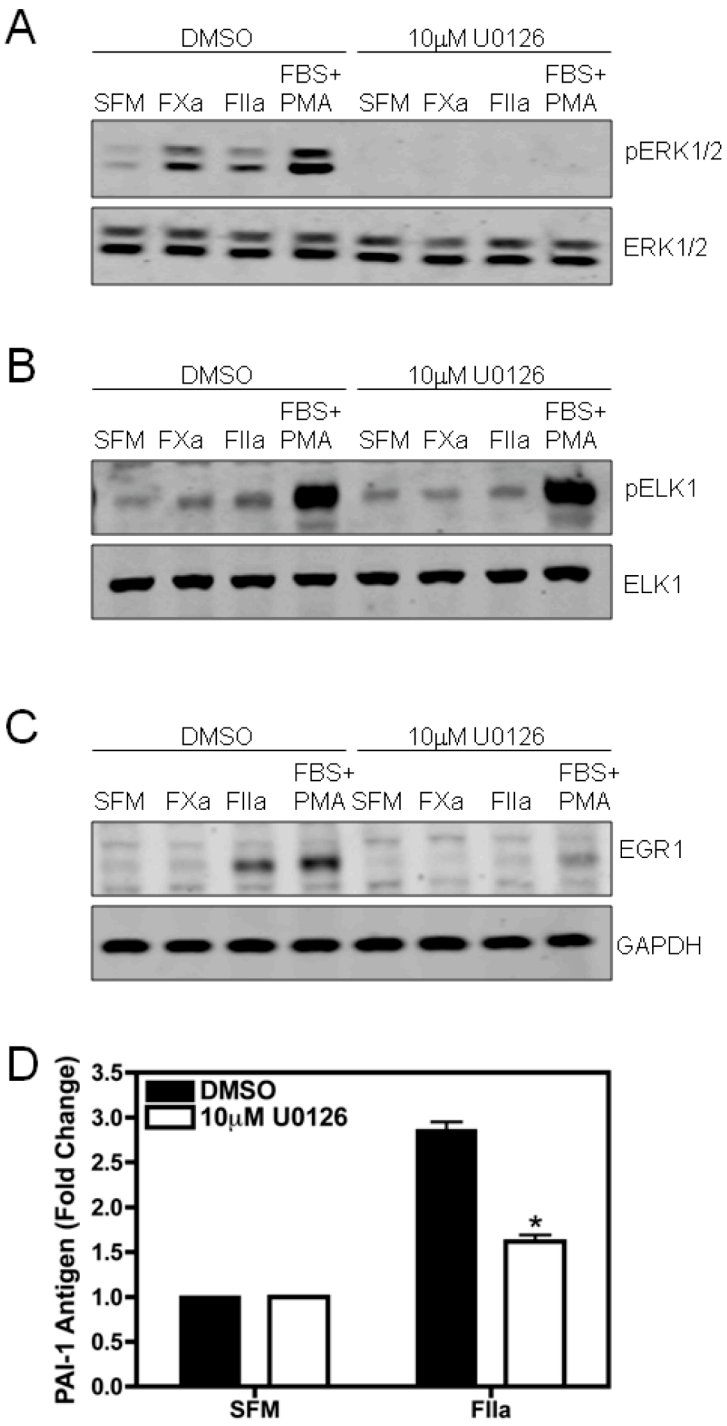

Figure 4.3. U0126 attenuates MAPK signaling and PAI-1 expression in thrombin stimulated 4T1 cells. Cells were pretreated with U0126 for 1 hour and then stimulated with 125nM FXa, 20nM thrombin (FIIa), or 10% FBS+200nM PMA (FBS+PMA) for 5 minutes. Membranes were probed for pERK1/2 and total ERK1/2 (A), pELK1 and total ELK1 (B), or EGR1 and GAPDH (C). (D) PAI-1 levels in the cell culture supernatant was measured by ELISA. Results are shown as mean ± SEM of four (A), three (C), and five (D) independent experiments. *P≤0.05 (DMSO versus 10μM U0126). The pELK1 blot is representative of two independent experiments.

DISCUSSION

The aim of this study was to determine the intracellular signaling pathways and transcription factors that regulate thrombin-induced PAI-1 gene expression in 4T1 cells. We examined the role of the NFκB and p42/p44 MAPK signaling pathways in thrombin-mediated PAI-1 expression. While there was no evidence of NFκB signaling, both FXa and thrombin increased the levels of pERK1/2 in 4T1 cells. We have previously demonstrated that thrombin-cleaved PAR-1 transactivated PAR-2 to induce PAI-1 expression in 4T1 cells[14]. Reducing either PAR-1 or PAR-2 expression in 4T1 cells decreased the levels of pERK1/2 in response to thrombin (data not shown). The U0126 compound prevents activated MEK from phosphorylating ERK1/2. U0126 inhibited phosphorylation of ERK1/2 and decreased PAI-1 protein levels. Taken together, the data indicate that thrombin activates PAR-1, which subsequently transactivates PAR-2, thereby inducing p42/p44 MAPK signaling.

The transcription factor ELK1 is a downstream target of pERK1/2. Phosphorylation of ELK1 increases its transcriptional activity by inducing a conformational change within the protein that allows for increased DNA binding activity and also by recruiting co-activators[31]. Both FXa and thrombin increased pELK1 levels in 4T1 cells. Interestingly, U0126 did not substantially decrease ELK1 phosphorylation in 4T1 cells treated with the positive control. This may reflect the fact that signaling pathways in addition to the p42/p44 MAPK pathway also induce ELK1 phosphorylation[29,31].

pELK1 induces transcription of the immediate early gene EGR1 by binding to the serum response element (SRE) in the EGR1 promoter[31,28]. Thrombin, but not FXa, increased EGR1 expression, indicating that FXa and thrombin activate different downstream signaling

pathways in 4T1 cells. In chick fibroblasts, thrombin induced the phosphorylation of ELK1[28]. The subsequent transcriptional activity of pELK1 in these cells was dramatically increased by the p300 co-activator. Furthermore, c-Jun N-terminal kinases (JNK) and p38 MAPK induced ELK1 phosphorylation in NIH3T3 fibroblasts, which resulted in EGR1 mRNA transcription[29]. It is possible that thrombin activates multiple intracellular signaling pathways and transcription factors, including p42/p44 MAPK, ELK1, and p300 that increase EGR1 mRNA and protein expression in 4T1 cells. Conversely, FXa activation of ERK1/2 and ELK1 is not sufficient for EGR1 expression, potentially due to a lack of co-activator recruitment or activation of supplemental transcription factors.

Alternatively, EGR1 is negatively regulated by nerve growth factor induced-A (NGFI-A) binding proteins 1 and 2 (NAB1 and NAB2)[32]. NAB1 is constitutively expressed whereas NAB2 is transiently expressed. NAB2 expression is induced by similar signaling stimuli as EGR1[32]. The observed FXa-dependent signaling may result in the expression of both EGR1 and NAB2, thereby inhibiting EGR1 expression in 4T1 cells. To fully understand the differential effects of FXa compared to thrombin, detailed studies of the EGR1 promoter region are necessary.

Using a PAR-2 agonist peptide, we show that PAR-2 activation increases EGR1 mRNA expression in 4T1 cells. Recently, a gene expression profile of PAR-1 and PAR-2 dependent transcripts in human kidney cells was compiled, revealing that EGR1 is downstream of PAR-2 activation[33]. This work independently validates our data. Furthermore, both FVIIa and FXa, presumably via PAR-2 activation, induced EGR1 mRNA expression[34]. Together, we propose a model in which thrombin activates PAR-1, which then transactivates PAR-2 resulting in ERK1/2 phosphorylation and subsequent ELK1

phosphorylation. In this model, pELK1 increases EGR1 gene transcription and protein expression thereby promoting PAI-1 expression (Figure 4.4).

In human breast cancer cells, ELK1 has been shown to bind the PAI-1 promoter directly, and upon epidermal growth factor (EGF) induced phosphorylation, promotes PAI-1 transcription[23]. Although ELK1 may indeed bind to the PAI-1 promoter, our data indicate that EGR1 induction is required for PAI-1 expression in the 4T1 mouse breast cancer cell line. The signaling pathways activated by EGF versus thrombin may account for the different roles ELK1 plays in the transcriptional regulation of PAI-1. Whether EGR1 serves as a crucial co-activator or as the major transcription factor regulating PAI-1 expression in 4T1 cells has yet to be determined.

Interestingly, preliminary data indicated that FXa activated ERK1/2 in the cells with reduced PAR-1 or PAR-2 expression, although some reduction in pERK1/2 levels was observed (data not shown). In addition to PAR-1 and/or PAR-2, effector cell protease receptor-1 (EPR1) is transmembrane receptor that serves as a FXa receptor and is expressed on cancer cells[35,36]. The FXa-induced phosphorylation of ERK1/2 and ELK1 that was not mediated by activation of PAR-1 or PAR-2 may indeed be a result of EPR1 activation. However, there are no available antibodies or inhibitory peptides that will inhibit murine EPR1.

In conclusion, PARs are cell surface receptors that, upon activation, mediate a variety of cellular effects. The mechanism of receptor activation dictates the intracellular signaling pathway(s) that will be utilized. The anti-fibrinolytic protein PAI-1 is expressed upon PAR-2 activation of 4T1 cells. Various signaling pathways regulate PAI-1 expression, many of which are induced by PAR-1 and PAR-2 activation. In this study, we demonstrate that the

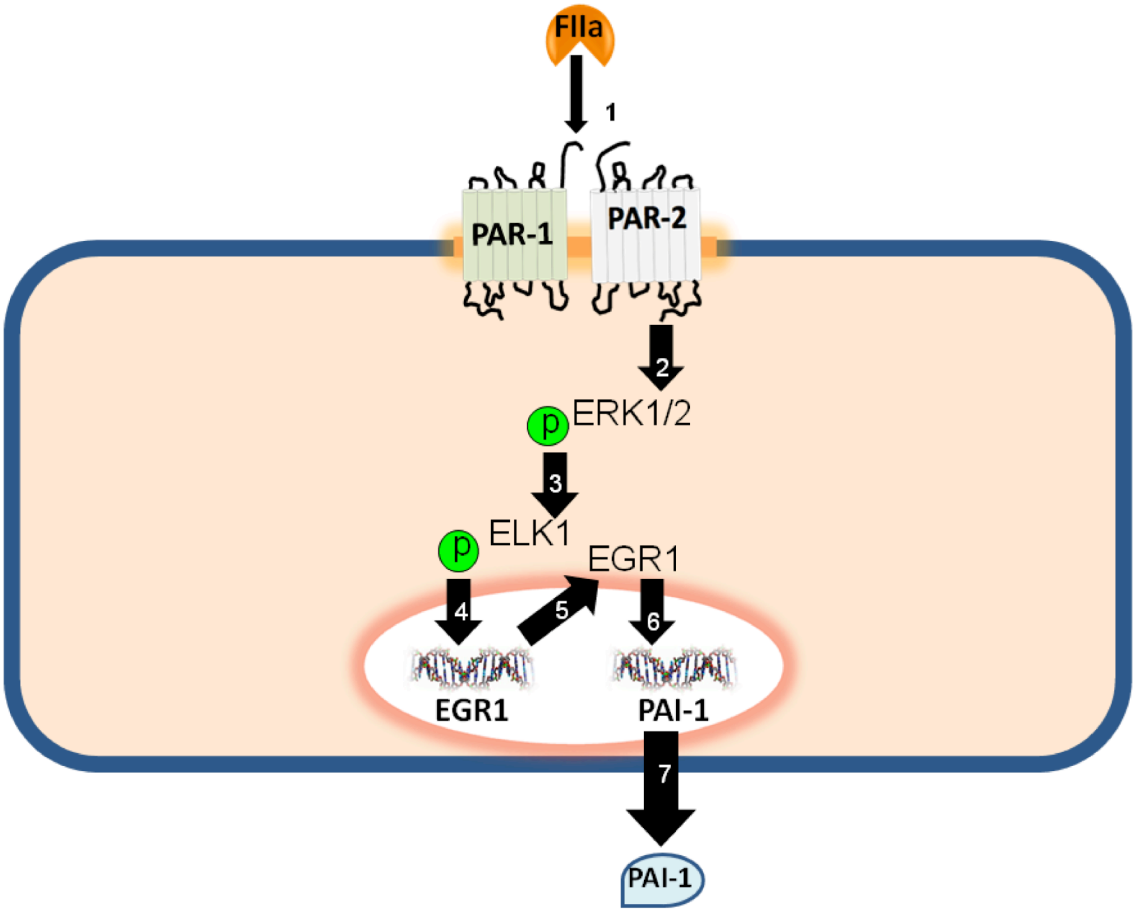

Figure 4.4. The proposed signaling mechanism driving thrombin-induced PAI-1 expression. (1) Thrombin activates PAR-1, which subsequently transactivates PAR-2. (2) Activation of PAR-2 results in phosphorylation of ERK1/2. (3) pERK1/2 phosphorylates ELK1. (4) pELK1 promotes EGR1 transcription. (5) EGR1 protein is translated. (6) EGR1 protein induces PAI-1 transcription. (7) PAI-1 is processed and released from the cells.

p42/p44 MAPK-ELK1-EGR1 pathway is required for thrombin-induced PAI-1 expression in metastatic murine breast cancer cells.

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CHAPTER V:

Discussion and Future Directions

In the human breast cancer cell line MDA-MB-231, exogenously added FVIIa binds to TF and activates PAR-2. This increases the migration and release of pro-angiogenic factors from the cells[1,2]. The initial goal of my research project was to analyze TF-FVIIa-PAR-2 signaling in non-metastatic (67NR) and metastatic (4T1) mouse breast tumor cell lines. 67NR cells only expressed PAR-1, while the 4T1 cells expressed both PAR-1 and PAR-2, making these cell lines appropriate models for this investigation. However, in contrast to the literature using human breast cancer cells, mouse FVIIa did not activate the 4T1 cells. I concluded that the relatively low levels of TF and/or PAR-2 expressed in the 4T1 cells did not sufficiently support TF-FVIIa-PAR-2 signaling. From these results, I altered the direction of my project to explore roles of FXa and thrombin-induced signaling by PAR-1 and PAR-2 in breast tumor progression using the 4T1 murine mammary adenocarcinoma model.

Activation of PAR-1 or PAR-2 induces the expression of uPA and PAI-1 in certain cancer cell lines[1,3]. uPA and PAI-1 regulate the fibrinolytic pathway and also have roles in tumor invasion, angiogenesis, and metastasis[4-7]. I investigated whether FXa, which activates PAR-1 and PAR-2, or thrombin, which activates PAR-1, could increase either uPA or PAI-1 expression in 4T1 cells *in vitro*. Two novel findings were made. First, FXa and thrombin activated PAR-1 resulting in a rapid release of stored uPA from inside the 4T1 cells. Thus, activation of PAR-1 induces uPA secretion. This observation was not specific to the 4T1 cells as I obtained similar results using two other murine breast cancer cell lines (168FARN and 4T07) and a murine pancreatic cancer cell line (PAN02). It would be interesting to further investigate if FXa and thrombin induce uPA secretion in non-transformed cells and to

determine if this PAR-1-dependent uPA secretory mechanism is applicable to human cell lines.

The second novel finding was that thrombin-activated PAR-1 transactivated PAR-2 to induce PAI-1 mRNA and protein expression. This is the first reported evidence that thrombin-induced PAI-1 is regulated by a PAR-1/PAR-2 complex. A more detailed investigation of the PAR-1/PAR-2 complex was not feasible due to limited reagent availability for murine PAR-2. For example, PAR-2 antibodies would have allowed me to determine if the PAR-1/PAR-2 complex was localized to lipid rafts or caveolae. The PAR-1 agonist peptide SFLLRNP-NH_2, corresponding to the tethered ligand sequence of human PAR-1, activates both human and murine PAR-2 *in vitro*[8]. Lack of anti-mouse PAR-2 antibodies directed towards the activation loop of the receptor prevented me from examining if the tethered ligand of thrombin-activated PAR-1 interacts with the activation loop of PAR-2. Furthermore, the transmembrane domain 4 of PAR-4 appears to interact with PAR-1 to form a PAR-1/PAR-4 complex (Dr. Marvin Nieman, personal communication). A similar interaction may occur in between PAR-1 and PAR-2 in 4T1 cells. To perform these experiments, antibodies against multiple mouse PAR-1 and mouse PAR-2 epitopes would need to be raised and mutated receptors would need to be generated.

To determine if activation of the PAR-1/PAR-2 complex was unique to thrombin, I incubated $4T1^{GFP}$, $4T1^{\Delta PAR\text{-}1}$, and $4T1^{\Delta PAR\text{-}2}$ cells with plasmin. Similar to thrombin, plasmin-induced uPA was PAR-1 dependent whereas PAI-1 induction required both PAR-1 and PAR-2 expression (Figure 5.1-A and B). These results reinforce my hypothesis stated in Chapter 3 that only non-membrane bound proteases, such as thrombin and plasmin, are capable of activating the PAR-1/PAR-2 complex, whereas membrane bound proteases, like

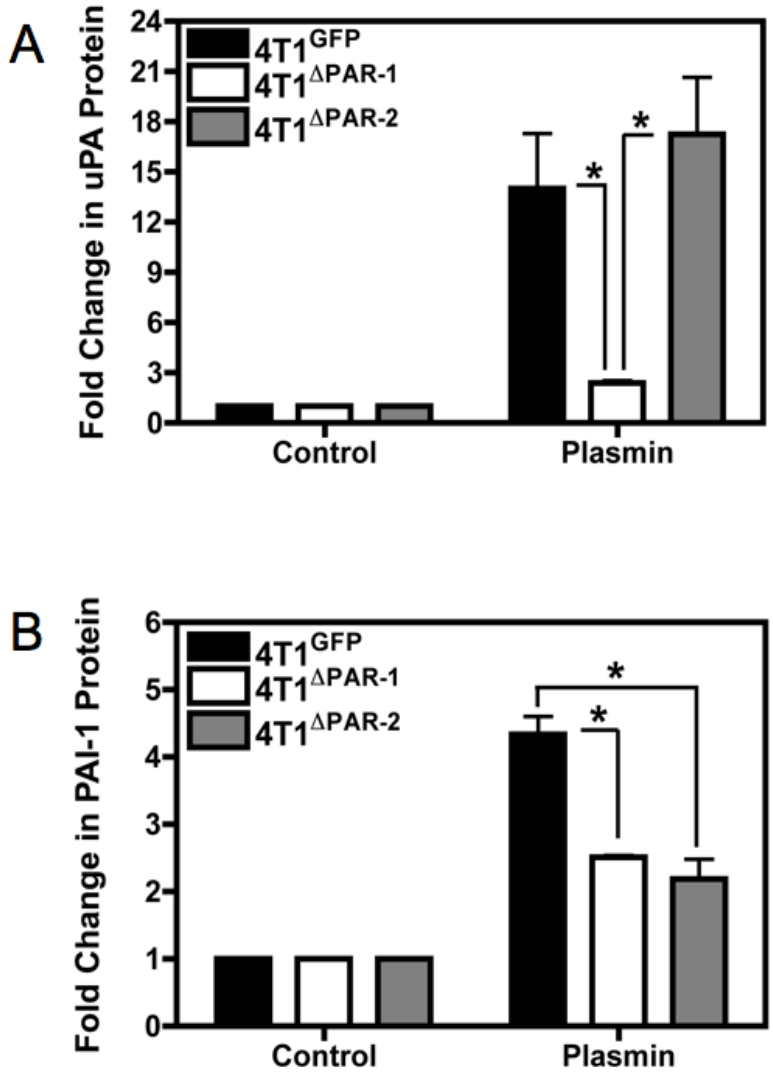

Figure 5.1. Plasmin induces uPA and PAI-1 in 4T1 cells. uPA protein (A) and PAI-1 protein (B) were measured by ELISA after 24 hour incubation with 100nM plasmin. Results are shown as mean ± SEM of three independent experiments. *P≤0.05.

FXa, are not. Additional experiments using membrane-bound proteases such as APC bound to endothelial protein C receptor (EPCR) or matriptase may be able to further confirm this hypothesis.

The signaling mechanism regulating thrombin-induced PAI-1 expression in 4T1 cells was also explored. I found that thrombin activates the ERK1/2-ELK1-EGR1 pathway and that the p42/p44 MAPK pathway is required for PAI-1 expression. Surprisingly, FXa increased the phosphorylation of ERK1/2 and ELK1 in $4T1^{GFP}$, $4T1^{\Delta PAR\text{-}1}$, and $4T1^{\Delta PAR\text{-}2}$ cells but FXa did not induce either EGR1 mRNA or protein expression. I propose that thrombin signaling was sufficient to recruit the necessary transcriptional co-factors for EGR1 expression while FXa was not. This may explain why thrombin, but not FXa, is capable of inducing PAI-1 expression in 4T1 cells. To further explore this possibility and to determine if EGR1 is the sole transcription factor needed for thrombin-induced PAI-1 expression in 4T1 cells, EGR1 would need to be silenced and PAI-1 promoter studies would have to be conducted.

Based on the literature and my *in vitro* data using 4T1 cells, I propose a model in which activation of PAR-1 induces the release of uPA, thus promoting sustained ECM proteolysis (Figure 5.2-A) as PAR-1 localized at the leading edge of invasive cancer cells[9]. Activation of PAR-1 by proteases, such as FXa or thrombin, results in the rapid release of uPA from 4T1 cells. uPA converts plasminogen to plasmin which then activates MMP-1[10]. Plasmin and MMP-1 activate PAR-1 thereby creating a positive feedback loop[11,12]. Additionally, plasmin and MMP-1 degrade the ECM, freeing and activating cell- or matrix-bound growth factors in the process[13]. The constant presence of these proteases and the kinetics of uPA secretion favors persistent ECM degradation and growth factor availability.

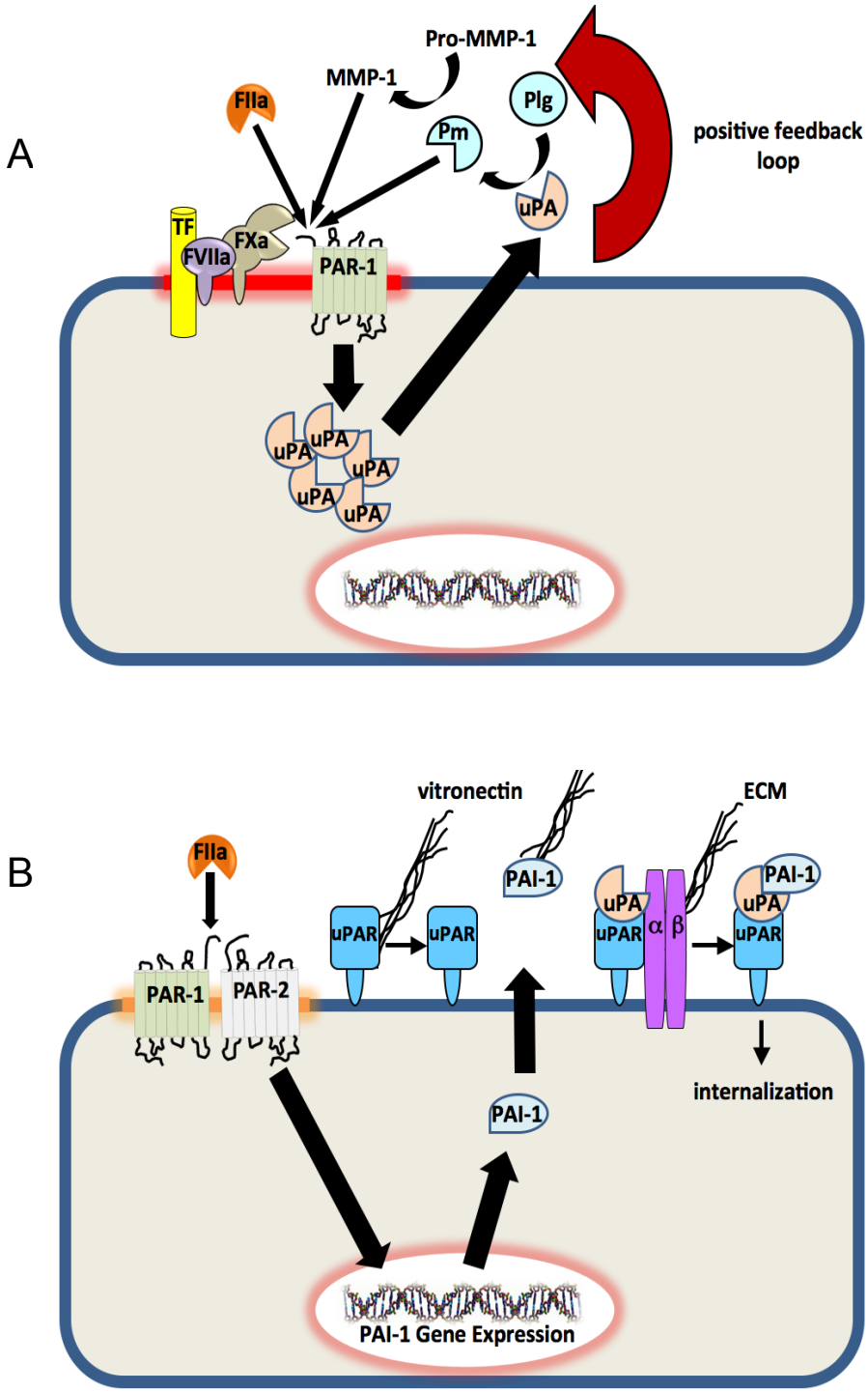

Figure 5.2. Proposed mechanism of how coagulation protease-mediated activation of PAR-1 or the PAR-1/PAR-2 complex promotes tumor invasion. (A) FXa or thrombin activates PAR-1, inducing the rapid release of stored uPA from 4T1 cells. uPA converts plasminogen (Plg) to plasmin (Pm) on the cell surface. Plasmin activates pro-MMP-1 to active MMP-1. Both plasmin and MMP-1 degrade the extracellular matrix and activate PAR-1 to release more uPA, creating a positive feedback loop. (B) Activation of the PAR-1/PAR-2 complex by thrombin increases PAI-1 expression. PAI-1 competes with uPAR for vitronectin binding and disrupts the uPA-uPAR complex binding to integrins (purple) by binding to uPA and internalizing the PAI-uPA-uPAR complex, thereby favoring cellular de-adhesion.

Activation of the PAR-1/PAR-2 complex also results in PAI-1 mRNA and protein expression. PAI-1 promotes cellular de-adhesion by competing with urokinase plasminogen activator receptor (uPAR) for vitronectin binding and by disrupting uPA-uPAR-integrin complexes via internalization of PAI-1-uPA-uPAR complexes (Figure 5.2-B)[14]. Rapid ECM degradation mediated by uPA activity, followed by PAI-1 induced cellular de-adhesion may further promote or sustain tumor cell invasion.

The data obtained from the *in vivo* experiments revealed that PAR-1 has a role in the growth of 4T1 cells and that PAR-2 is involved in hematogenous metastasis in the 4T1 breast tumor model. The mechanisms underlying these results were not delineated. There were no significant differences in uPA or PAI-1 levels between plasma samples from mice implanted with $4T1^{GFP}$, $4T1^{\Delta PAR-1}$, or $4T1^{\Delta PAR-2}$ cells. Lower levels of uPA were detected in the plasma samples from tumor bearing mice in comparison to control mice while the opposite was true for PAI-1. A possible explanation for the decreased uPA levels in tumor bearing mice is that the majority of uPA was bound to the surface of the 4T1 cells and subsequently internalized as part of the PAI-1-uPA-uPAR complex. Laser capture microdissection of $4T1^{GFP}$, $4T1^{\Delta PAR-1}$, or $4T1^{\Delta PAR-2}$ tumor cells followed real-time PCR could be used to determine if reducing PAR-1 or PAR-2 expression in 4T1 cells has an effect on PAI-1 expression in these cells *in vivo*. This approach might not be useful in detecting changes in uPA expression as my *in vitro* data indicates that activating PAR-1 or PAR-2 does not regulate uPA transcription. An alternative approach is to grow $4T1^{GFP}$, $4T1^{\Delta PAR-1}$, and $4T1^{\Delta PAR-2}$ tumors in either uPA or PAI-1 deficient animals and determine uPA and PAI-1 protein levels in the plasma and in stained tumor sections.

Activation of PAR-1 or PAR-2 mediates inflammation[1,15]. I investigated the effects of reducing PAR-1 or PAR-2 expression in tumor cells on *in vivo* cytokine levels. Surprisingly, of the 62 cytokines assayed, reducing PAR-1 or PAR-2 expression significantly impacted the expression of only one cytokine, granulocyte colony stimulating factor (GCSF). Tumor-derived GCSF recruits tumor associated neutrophils (TANs), myeloid suppressor cells, and endothelial progenitor cells, thereby promoting angiogenesis, invasion, and immunosuppression[16-20]. *In vivo,* plasma GCSF levels were significantly reduced in the samples from mice with $4T1^{\Delta PAR\text{-}2}$ tumors compared to mice with $4T1^{GFP}$ or $4T1^{\Delta PAR\text{-}1}$ tumors. Low levels of GCSF were detected in the plasma of control mice. *In vitro*, activation of PAR-1 or PAR-2 increased GCSF in the culture supernatant of 4T1 cells. Staining tumor sections for vascular density, neutrophils, and $CD11b^{+}/GR1^{+}$ myeloid suppressor cells may yield useful information in further characterizing the role of PAR-2-dependent GCSF expression in the 4T1 breast tumor model. Additionally, reducing PAR-2 expression in 4T1 cells decreased both lung metastasis and GCSF levels *in vivo*. Using a neutralizing antibody against GCSF *in vivo* could help determine if GCSF is involved in the hematogenous metastasis of 4T1 cells.

TF and the downstream coagulation proteases contribute to malignancy[21]. PARs are the cellular substrates for FVIIa, FXa, and thrombin. Inhibition of TF could reduce PAR activation by FVIIa, FXa, or thrombin. Inhibition of FVIIa, FXa, or thrombin has shown some promise in experimental models of melanoma, colorectal cancer, and glioblastoma [22-24]. However, targeting TF, FVIIa, FXa, or thrombin may have undesired hemorrhagic consequences. Despite these findings, there are numerous proteases present in the tumor stroma that could activate PAR-1 and PAR-2 in the absence of TF and coagulation proteases.

Targeting PAR-1 and/or PAR-2 seems promising; however, these receptors regulate overlapping sets of genes[1,25]. Using the $4T1^{GFP}$, $4T1^{\Delta PAR\text{-}1}$, and $4T1^{\Delta PAR\text{-}2}$ cell lines to create expression profiles of genes induced by the activation of PAR-1, PAR-2, or by PAR-1 transactivation of PAR-2 would elucidate the potential druggability of these receptors. Creating such comprehensive gene expression profiles will require the use of a variety of PAR-1 and PAR-2 agonists. Nevertheless, specifically targeting tumor PAR-1 and/or PAR-2 may be difficult since both are ubiquitously expressed.

The goal of my project was to examine if coagulation protease activation of PAR-1 or PAR-2 expressed by 4T1 breast cancer cells modulates the expression of components of the plasminogen activator system. The data presented here is not an exhaustive investigation of the entire plasminogen activator system as I focused specifically on uPA and PAI-1. uPAR and tissue plasminogen activator (tPA) also have roles in promoting the malignant phenotype of cancer cells[26-28]. It would be interesting to examine if activation of PAR-1 and/or PAR-2 regulates uPAR or tPA expression in the 4T1 cells and the effects this may have on breast tumor progression.

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APPENDIX I:

Effect of Reducing Protease-Activated Receptor-1 or -2 Expression in Breast Tumor Cells on Tumor Growth, Metastasis, and Cytokine Production *In Vivo*

INTRODUCTION

Protease activated receptor-1 (PAR-1) and PAR-2, members of the G-protein coupled receptor family, have been implicated in promoting tumor growth, survival, angiogenesis, and invasion[1-3]. PAR-1 is activated by the coagulation proteases FVIIa, FXa and thrombin, in addition to matrix metalloproteinase-1 (MMP-1), plasmin, and activated protein C (APC)[4-8]. PAR-2 is also activated by coagulation proteases, namely FVIIa and FXa[9]. Other PAR-2 agonists include trypsin, matriptase, and mast cell tryptase[10]. We have published that PAR-1 and PAR-2 positively regulate the expression of urokinase plasminogen activator (uPA) and its inhibitor, plasminogen activator inhibitor-1 (PAI-1)[11]. Inflammatory cytokines are also expressed in response PAR-1 or PAR-2 activation[12,13]. The goal of this study was to determine the effects of reducing tumor cell PAR expression on tumor growth and metastasis as well as the impact on uPA, PAI-1, and inflammatory cytokine levels *in vivo*.

MATERIALS AND METHODS

Reagents.

Sterile syringes and needles were obtained from BD Biosciences. Sodium dodecyl sulfate (SDS), glycerol, tris(hydroxymethyl)aminomethane (Tris), phosphate buffered saline (PBS), trypan blue, penicillin/streptomycin, and sodium citrate were purchased from Sigma-Aldrich. Complete protease inhibitor cocktail tablets and phosphatase inhibitors were obtained from Roche. Buffered 10% formalin pH 6.8-7.2 was purchased from VWR. Bouin's fixative was purchased from Ricca Chemical Company. PAR-1 and PAR-2 agonist peptides were obtained from Calbiochem. Purified human coagulation proteases factor Xa (FXa) and alpha-thrombin (FIIa) were purchased from Haematologic Technologies Inc.

Recombinant mouse factor VIIa (mFVIIa) was provided by Dr. Lars Petersen (Novo Nordisk).

Animals.

All animal experiments were performed in accordance with the guidelines of the institutional animal care and use committee (IACUC) at the University of North Carolina at Chapel Hill (UNC-CH). 6-8 week old female Balb/c mice were obtained from Charles River Laboratories. The mice were housed and maintained by the Division of Laboratory Animal Medicine (UNC-CH). Mice were housed in 12-hour day/night cycles.

Cell culture.

The highly metastatic 4T1 murine mammary adenocarcinoma cell line was obtained from Dr Fred Miller (Michigan Cancer Foundation). $4T1^{GFP}$, $4T1^{\Delta PAR\text{-}1}$, and $4T1^{\Delta PAR\text{-}2}$ cell lines were generated as previously described[11]. Cells lines were maintained in minimal essential media (MEM)-alpha (Gibco) supplemented with 10% fetal bovine serum (Omega Scientific), and 1% penicillin/streptomycin. Prior to implantation, cells were detached from the flasks with Versene (UNC-CH Tissue Culture Facility) and a sub-sampling was tested for viability by trypan blue exclusion.

***In vivo* tumor studies.**

Tumor implantation. Animal procedures were performed using sterile conditions by the Animal Studies Core Facility (UNC-CH). The recipient mice were anesthetized and a small midline incision was made in the lower abdomen and then angled toward the left

inguinal mammary fat pad. Using a sterile syringe with a 27-guage needle, 100μL of a single cell suspension in PBS was injected into the 4th mammary fat pad. Sterile PBS was injected into the control mice. The incision was closed with wound clips. Once fully healed, the wound clips were removed.

Tumor growth rates and weight. To determine the growth rate of 4T1^{GFP}, 4T1$^{\Delta PAR\text{-}1}$, or 4T1$^{\Delta PAR\text{-}2}$ tumors, 50,000 cells were implanted into the 4th mammary fat pad. Tumor growth was monitored for 22 days by taking digital caliper measurements of the length and width of each tumor. The tumor volume was calculated using the following equation: *Volume (cm^3) = ½ Length x Width^2*. At the conclusion of the experiment, the tumors were carefully excised, rinsed in ice cold PBS, and weighed.

Quantification of spontaneous pulmonary metastasis. For all spontaneous metastasis experiments, 100,000 4T1^{GFP}, 4T1$^{\Delta PAR\text{-}1}$, or 4T1$^{\Delta PAR\text{-}2}$ cells were implanted into the 4th mammary fat pad and allowed to grow for 21 days. At the conclusion of the experiment, the lungs were removed and placed in Bouin's fixative. The surface metastatic nodules on the lungs were counted.

Exsanguination and plasma preparation.

At the time of sacrifice, the animals were anesthetized and exsanguinated via the inferior vena cava. 500μL of blood was drawn into a sterile syringe containing 50μL of sodium citrate as an anticoagulant. The blood was placed on ice then centrifuged at 4,000 x g for 15 minutes at 4^{o}C. The plasma was stored at -80^{o}C until use.

uPA and PAI-1 enzyme-linked immunosorbent assays.

Confluent 4T1 cell monolayers were serum starved for 16 hours in serum free media (SFM) composed of MEM-alpha (Gibco) supplemented with 1% penicillin/streptomycin. Following starvation, fresh SFM containing either PAR-1 agonist peptide, PAR-2 agonist peptide, mFVIIa, FXa, or thrombin were added to the wells. The cell culture supernatant was collected after 24 hours, centrifuged at 5,000 x *g* for 5 minutes at 4^{o}C to remove cellular debris, and frozen at -20^{o}C. An enzyme-linked immunosorbent assay (ELISA) was used to determine the levels of uPA, PAI-1, (Molecular Innovations) and granulocyte colony-stimulating factor (GCSF; R&D Systems) in the plasma samples and cell culture supernatants.

Cytokine array.

Plasma samples from control mice, $4T1^{GFP}$, $4T1^{\Delta PAR\text{-}1}$, or $4T1^{\Delta PAR\text{-}2}$ tumor bearing mice were pooled according to their respective cohorts. Each pooled plasma sample was added to an individual well of the RayBio Mouse Cytokine Antibody Array G Series 3 (RayBiotech) and the experiment was performed as per the manufacturers protocol with minor alterations. A 1:2,000 dilution of IRDye 680 Streptavidin (LI-COR Biosciences) was used for detection and the slides were scanned and quantified using the Odyssey Infrared Imaging System (LI-COR Biosciences).

Statistics.

GraphPad Prism 4 for Mac (GraphPad Software) was used to perform statistical analyses. All data are presented as means ± standard error of the mean (SEM). Data sets

were analyzed using a two-tailed students t-test. Alternatively, a two-way analysis of variance (ANOVA) with a Bonferroni post-hoc analysis was utilized when indicated. Grubb's test was performed to determine statistical outliers. $P \leq 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Decreasing PAR-1 expression in 4T1 cells reduces breast tumor growth.

To determine if PAR-1 or PAR-2 expression contributes to growth of 4T1 tumors, $4T1^{GFP}$, $4T1^{\Delta PAR\text{-}1}$, or $4T1^{\Delta PAR\text{-}2}$ cells were implanted into the mammary fat pad of immunocompetent female Balb/c mice. The growth rate and final weight of $4T1^{\Delta PAR\text{-}1}$ tumors was decreased by roughly 40% in comparison to that of $4T1^{GFP}$ and $4T1^{\Delta PAR\text{-}2}$ tumors (Figure A.1). These data are consistent with the literature which suggests that thrombin, a PAR-1 agonist, functions as a tumor growth factor[14]. Additionally, dabigatran, a direct thrombin inhibitor, reduced the growth of 4T1 cells both *in vitro* and *in vivo,* presumably by reducing the activation of PAR-1[15]. Decreasing PAR-2 expression in 4T1 cells did not effect tumor growth (Figure A1.1). Together, the data suggest that PAR-1 activation promotes the growth of 4T1 breast tumor cells *in vivo.*

Decreasing PAR-2 expression in 4T1 cells reduces hematogenous metastasis.

4T1 cells spontaneously metastasize from the mammary fat pad to distant sites including the lymph nodes, lung, liver, bone, and brain[16,17]. Silencing either PAR-1 or PAR-2 expression in 4T1 cells reduced the extent to which the cells hematogenously metastasized to the lungs (Figure A1.2). The lungs of mice implanted with $4T1^{\Delta PAR\text{-}1}$ or $4T1^{\Delta PAR\text{-}2}$ tumors

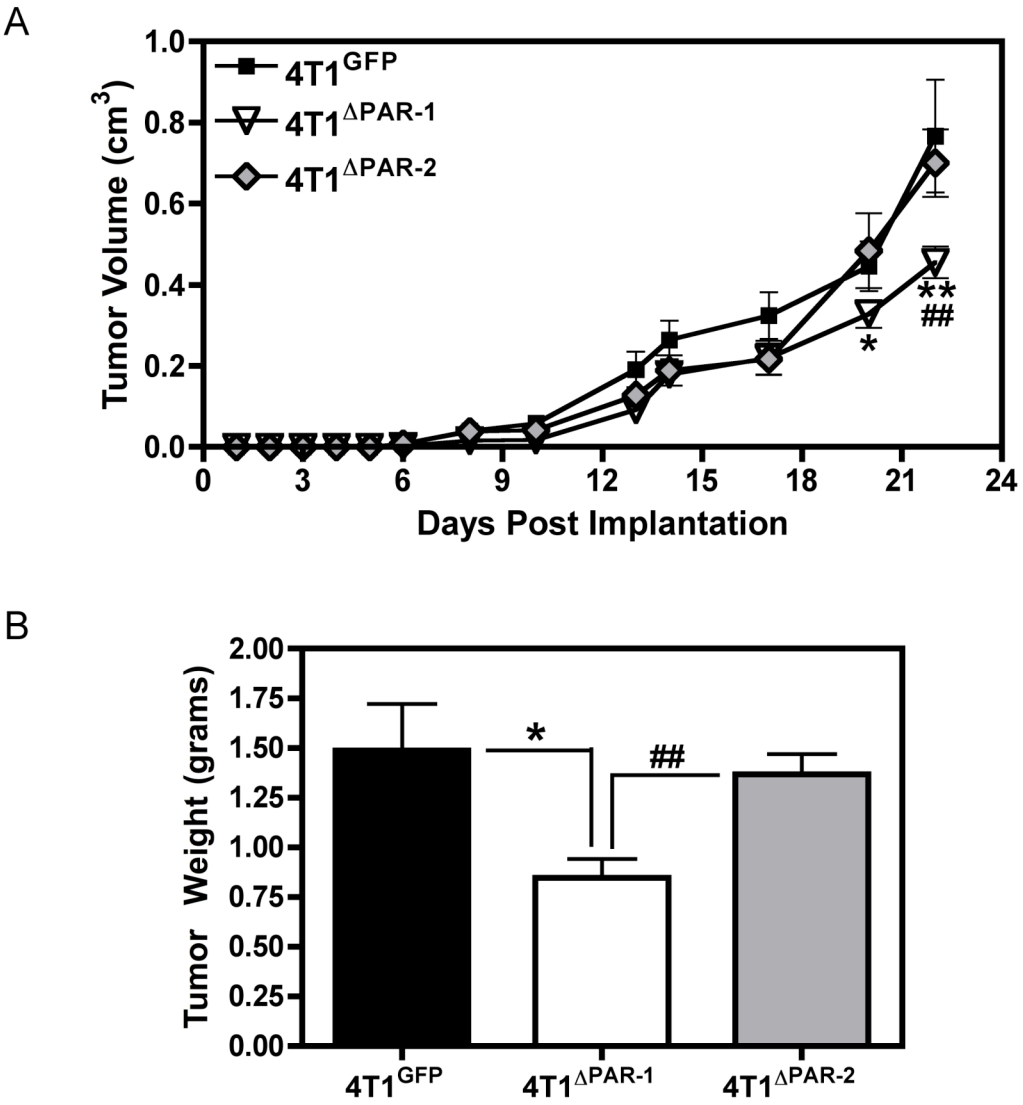

Figure A1.1. Silencing PAR-1 decreased tumor growth *in vivo*. (A) Tumor growth rate of $4T1^{GFP}$, $4T1^{\Delta PAR-1}$, or $4T1^{\Delta PAR-2}$ tumors. Mice were implanted with 50,000 $4T1^{GFP}$ (n=5 mice), $4T1^{\Delta PAR-1}$ (n=7 mice), or $4T1^{\Delta PAR-2}$ (n=5 mice) into the 4th mammary fat pad. Digital caliper measurements were recorded and used to calculate the tumor volume using the following ellipsoid equation: *Volume* (cm^3) = ½ *Length x Width*^{2}. Error bars represent the SEM. P-values were calculated using a two-way ANOVA with a Bonferroni post-hoc analysis. * P≤0.05 and ** P≤0.001 ($4T1^{GFP}$ versus $4T1^{\Delta PAR-1}$), ## P≤0.001 ($4T1^{\Delta PAR-2}$ versus $4T1^{\Delta PAR-1}$). (B) Tumor weight at time of sacrifice. $4T1^{GFP}$ (n=5), $4T1^{\Delta PAR-1}$ (n=7), and $4T1^{\Delta PAR-2}$ (n=5) tumors were excised and weighed. . Error bars represent the SEM. P-values were calculated using a two-tailed students t-test. * P≤0.05 ($4T1^{GFP}$ versus $4T1^{\Delta PAR-1}$), ## P≤0.001 ($4T1^{\Delta PAR-2}$ versus $4T1^{\Delta PAR-1}$).

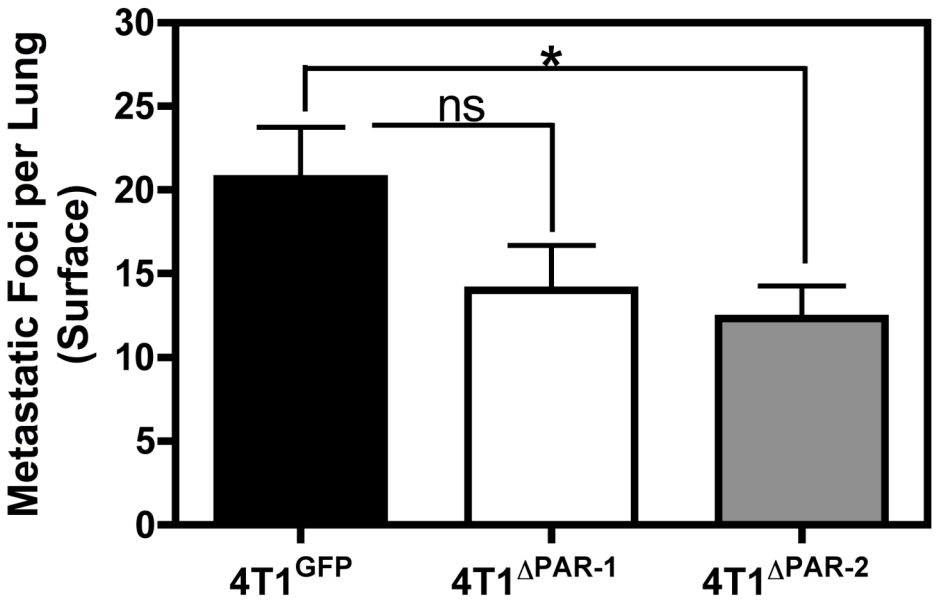

Figure A1.2. Reducing PAR-2 expression in tumor cells decreases spontaneous lung metastasis. Mice were implanted with 100,000 $4T1^{GFP}$ (n=6 mice), $4T1^{\Delta PAR\text{-}1}$ (n=5 mice), or $4T1^{\Delta PAR\text{-}2}$ (n=6 mice) into the 4th mammary fat pad. At the time of sacrifice, the lungs from each mouse were excised, rinsed in PBS, and fixed in Bouin's fixative. The visible metastatic nodules on the surface of each lung were counted in duplicate and averaged. Error bars represent the SEM. * $P \leq 0.05$ ($4T1^{GFP}$ versus $4T1^{\Delta PAR\text{-}2}$). One statistically significant outlier per tumor cohort was excluded using Grubb's test with a P-value of $P \leq 0.05$.

had fewer metastatic foci when compared to the lungs of mice with $4T1^{GFP}$ tumors. However, only the $4T1^{\Delta PAR\text{-}2}$ tumors displayed a statistically significant reduction in metastasis. This data suggests that PAR-2 contributes to tumor metastasis in the 4T1 breast tumor model. *In vitro,* activation of PAR-1 and PAR-2 regulate the expression of overlapping sets of genes[12,13]. This may also be true in 4T1 cells *in vivo*. An attempt to silence both PAR-1 and PAR-2 in the same cell was unsuccessful (data not shown).

Reducing PAR-1 or PAR-2 expression in 4T1 cells does not alter plasma uPA or PAI-1 levels.

To examine if reducing tumor cell PAR-1 or PAR-2 expression affects uPA or PAI-1 levels *in vivo*, plasma from control mice, $4T1^{GFP}$, $4T1^{\Delta PAR\text{-}1}$, or $4T1^{\Delta PAR\text{-}2}$ tumor bearing mice was analyzed by ELISA. Similar levels of uPA were detected in the plasma of mice implanted with $4T1^{GFP}$, $4T1^{\Delta PAR\text{-}1}$, or $4T1^{\Delta PAR\text{-}2}$ tumors (Figure A1.3-A). Interestingly, significantly more circulating uPA was present in the plasma from control (no tumor) mice in comparison to that of tumor bearing mice. Plasma PAI-1 levels were increased in tumor bearing mice in comparison to plasma from the control (no tumor) mice (Figure A1.3-B). However, there were no significant differences in the plasma PAI-1 levels between the cohorts of tumor bearing mice.

Our results suggest that reducing the expression of PAR-1 or PAR-2 in 4T1 cells does not impact the plasma levels of uPA or PAI-1 in tumor bearing mice. There are a variety of cell types that comprise breast tumors, including fibroblasts, endothelial cells, adipocytes, macrophages, and neutrophils. All of these cell types are known to express uPA and PAI-1[18-21]. This experiment did not specifically examine the cellular source of uPA and PAI-1.

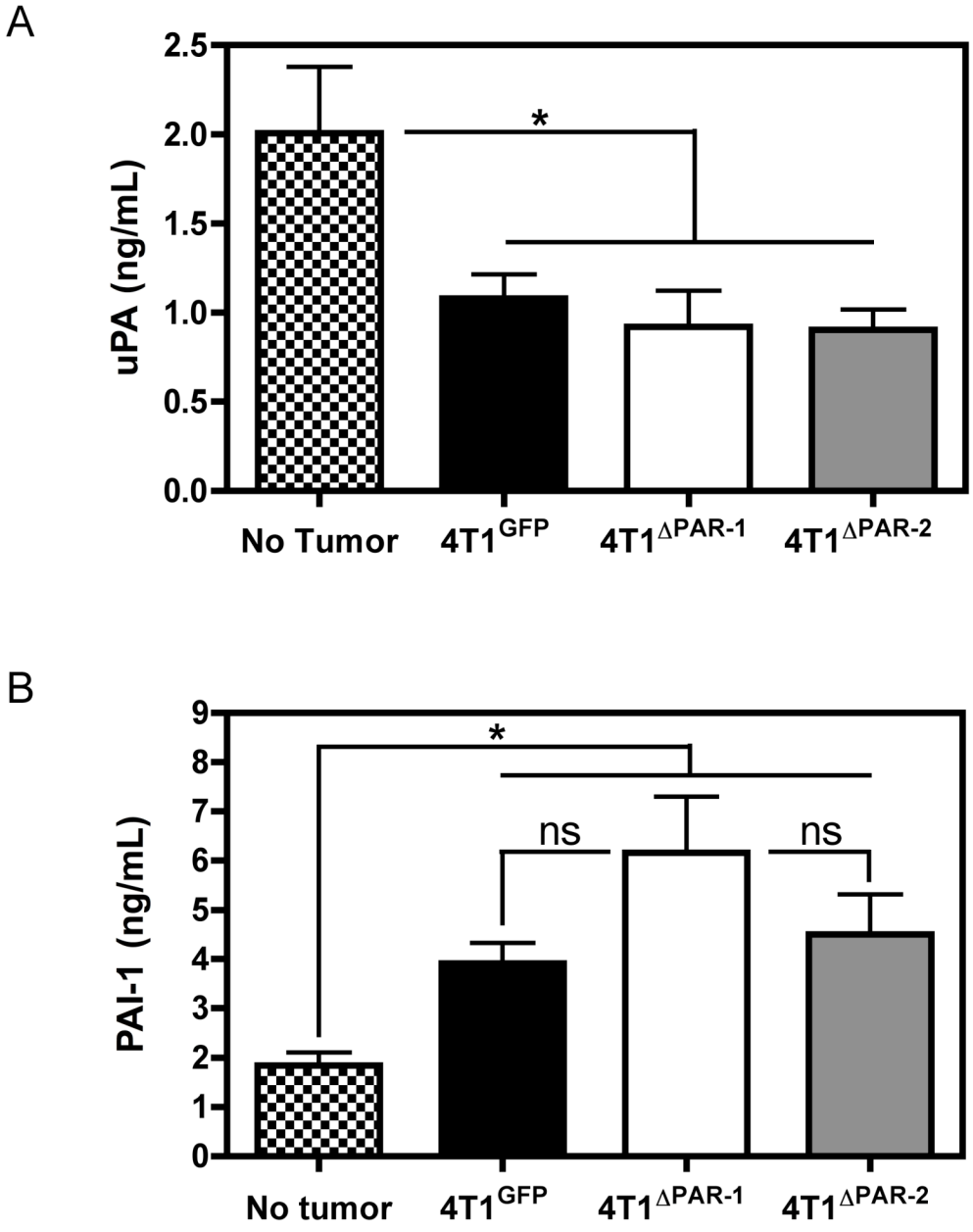

Figure A1.3. Determination of uPA and PAI-1 levels *in vivo*. Quantification of plasma levels of uPA (A) and PAI-1 (B) by ELISA. Plasma samples were taken from control mice (n=4), $4T1^{GFP}$ (n=7), $4T1^{\Delta PAR\text{-}1}$ (n=6), and $4T1^{\Delta PAR\text{-}2}$ (n=7). Error bars represent the SEM. * $P \leq 0.05$, ns (not significant).

Therefore, a different approach is needed to truly determine the effects of tumor cell PAR-1 and PAR-2 expression on uPA and PAI-1 *in vivo*. For example, implanting the $4T1^{GFP}$, $4T1^{\Delta PAR\text{-}1}$, and $4T1^{\Delta PAR\text{-}2}$ cells into uPA or PAI-1 deficient mice.

Reducing PAR-2 expression in 4T1 cells decreases GCSF levels *in vivo* and *in vitro*.

To examine if PAR-1 or PAR-2 activation contributes to inflammatory cytokine production, pooled plasma from each cohort of mice was assayed for 62 different cytokines using a cytokine antibody array. Reducing either PAR-1 or PAR-2 expression in the tumor cells resulted in decreased plasma levels of GCSF, granulocyte macrophage colony-stimulating factor (GM-CSF), chemokine (C-X-C motif) ligand-16 (CXCL-16), and interleukin-12 in comparison to plasma from mice with $4T1^{GFP}$ tumors (Figure A1.4-A). GCSF was further investigated due to its robust signal intensity and the recent reports that this chemokine promotes tumor aggressiveness[22-24].

To confirm the cytokine array data, plasma GCSF levels were measured by ELISA. Plasma levels of GCSF from each cohort of tumor bearing mice were significantly elevated in comparison to control mice (Figure A1.4-B). Within the tumor cohort, a reduction in GCSF was observed in the plasma of mice implanted with $4T1^{\Delta PAR\text{-}2}$ tumors compared with mice implanted with $4T1^{GFP}$ or $4T1^{\Delta PAR\text{-}1}$ tumors. This data suggest that expression of PAR-2 on tumor cells plays a role in the *in vivo* production of GCSF in the 4T1 breast cancer model.

To extend these observations, 4T1 cells were stimulated with increasing concentrations of PAR-1 or PAR-2 specific agonist peptides *in vitro*. While both PAR-1 and

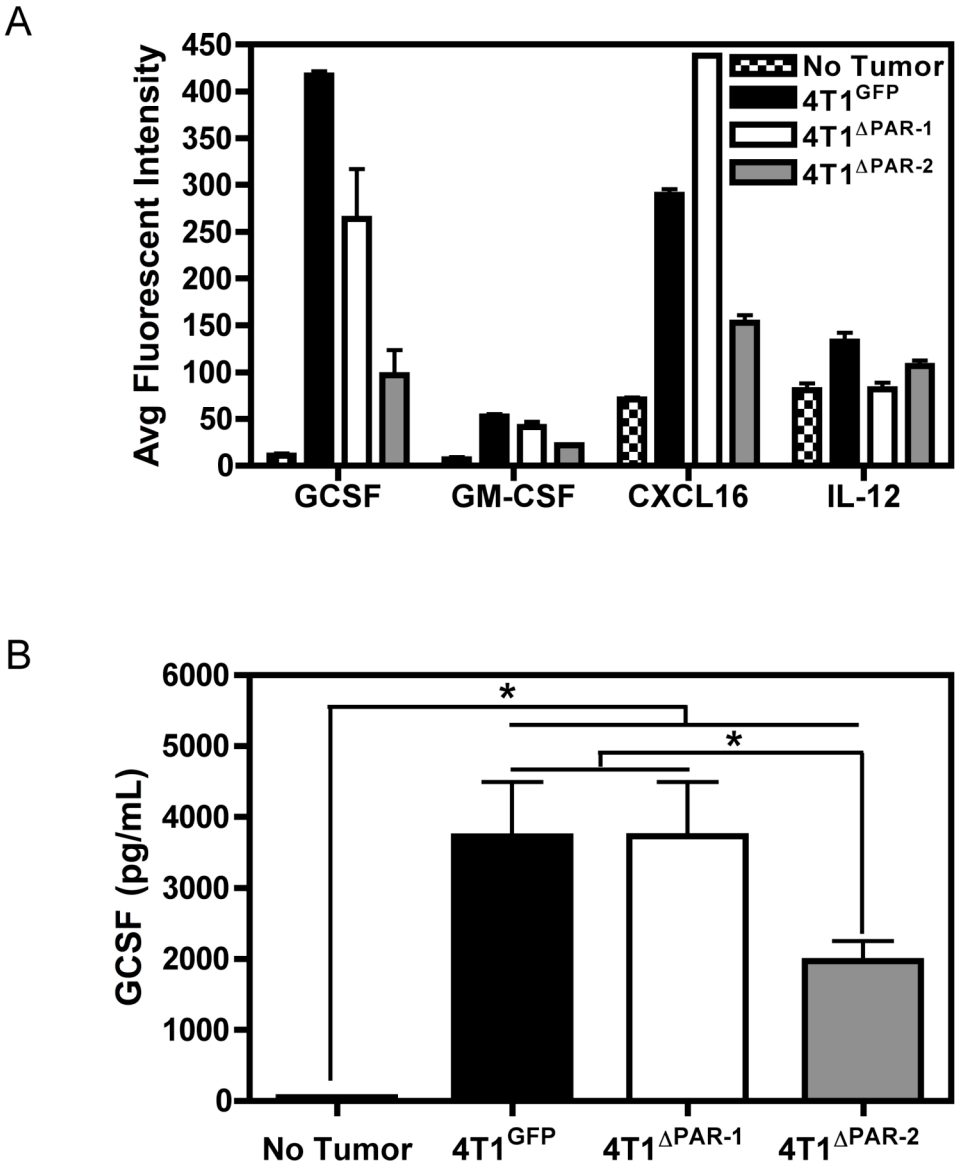

Figure A1.4. Silencing PAR-2 expression alters cytokine production *in vivo*. (A) The cytokine profile was generated using pooled plasma from each respective cohort of mice. Cytokine antibodies were spotted in duplicate on the slide. Error bars represent the SEM of duplicate antibody spots. (B) An ELISA was used to determine the levels of GCSF in the individual plasma samples. Control mice (n=4), $4T1^{GFP}$ (n=7), $4T1^{\Delta PAR\text{-}1}$ (n=6), and $4T1^{\Delta PAR\text{-}2}$ (n=7). Error bars represent the SEM. * $P \leq 0.05$.

PAR-2 agonist peptides increased the amount of GCSF in the culture supernatant of 4T1 cells, a stronger induction was observed with the PAR-2 agonist peptide (Figure A1.5-A). It has been demonstrated that agonist peptide activation of PARs results in different cellular responses compared with proteolytic activation[10]. Aware of this, 4T1 cells were stimulated with FVIIa, FXa, or thrombin (FIIa). Incubating 4T1 cells with mFVIIa did not induce GCSF *in vitro* (data not shown). FXa modestly increased the amount of GCSF in the culture supernatant while thrombin resulted in a more pronounced accumulation of GCSF (Figure A.5-B). Incubating $4T1^{GFP}$, $4T1^{\Delta PAR\text{-}1}$, and $4T1^{\Delta PAR\text{-}2}$ cells with FXa or thrombin revealed that these coagulation proteases activate PAR-1 to induce GCSF (Figure A.6). This data does not exclude PAR-2 from inducing GCSF *in vitro* because all of the PAR-2 proteolytic agonists were not examined *in vitro*. In addition to FVIIa and FXa, PAR-2 is activated by, mast cell tryptase, kallikreins, and matriptase, all of which are present in the tumor microenvironment[25-28].

We found a new relationship between PAR activation and GCSF expression. GCSF is a granulocyte chemoattractant. Of the granulocyte lineage, neutrophils have recently been implicated in tumor progression. Tumor associated neutrophils (TANs) are thought to contribute to tumor progression by favoring angiogenesis and releasing extracellular matrix degrading proteases[29,30]. Furthermore, GCSF also recruits endothelial progenitor cells and pro-angiogenic myeloid-derived suppressor cells[22,23]. This report provides preliminary data that merit further exploration into the role of PAR-induced GCSF and its effects on tumor growth and metastasis.

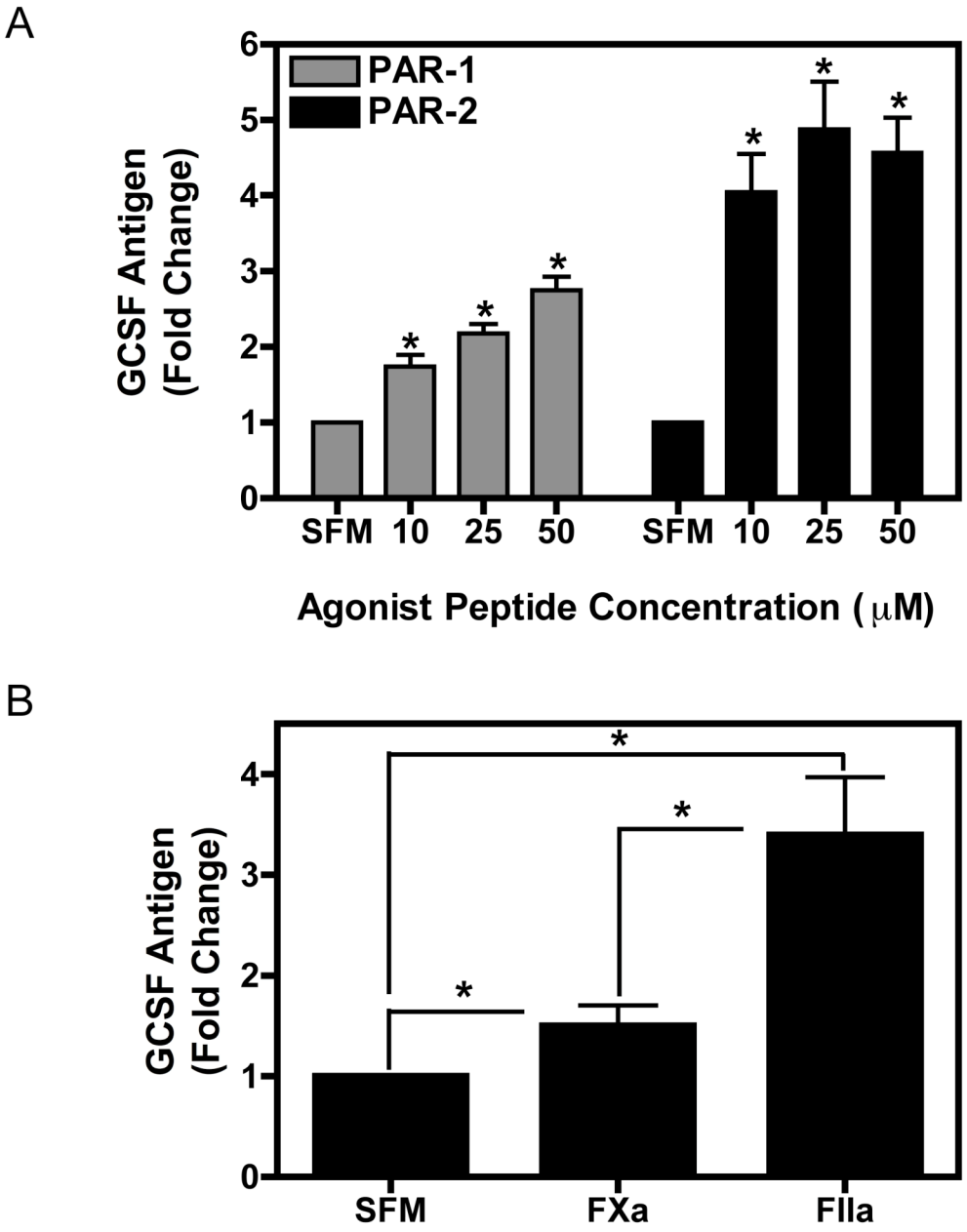

Figure A1.5. Activation of PAR-1 and PAR-2 induces GCSF *in vitro*. 4T1 cells were grown to confluence and starved in serum free media (SFM) for 16 hours before being stimulated with PAR-1 or PAR-2 agonist peptides (A) or 125nM FXa or 20nM thrombin (B) for 24 hours. GCSF levels in the cell culture supernatants were measured by ELISA. Results are shown as mean ± SEM of at least three independent experiments. *P≤0.05.

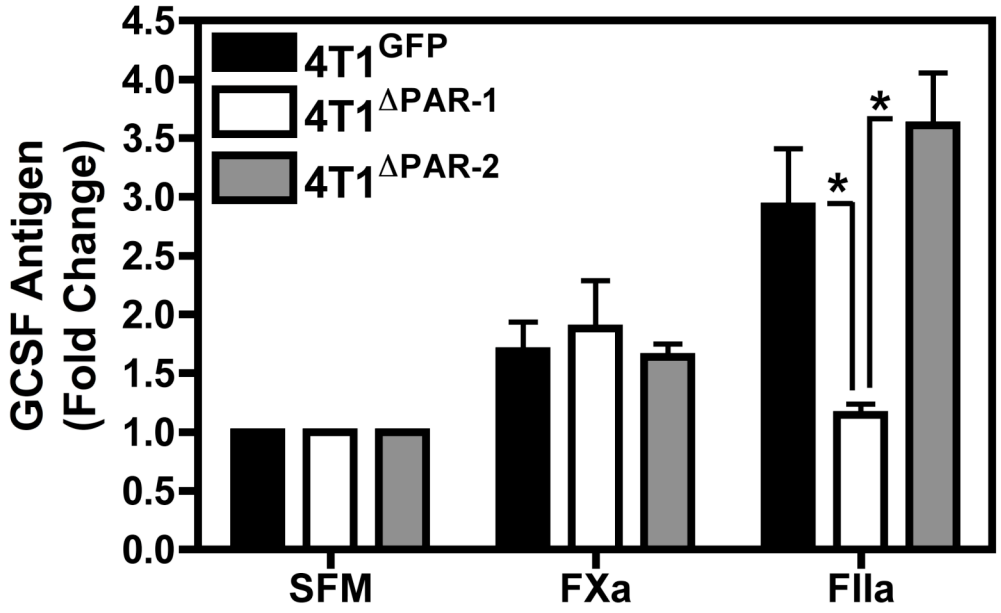

Figure A1.6. Thrombin increases GCSF expression in a PAR-1-dependent manner. Confluent $4T1^{GFP}$, $4T1^{\Delta PAR\text{-}1}$, and $4T1^{\Delta PAR\text{-}2}$ cells were starved for 16 hours then treated with 125nM FXa or 20nM thrombin (FIIa) for an additional 24 hours. GCSF levels in the culture supernatant were measured by ELISA. Results are shown as mean ± SEM of at least three independent experiments. *P≤0.05.

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APPENDIX II:

Tumors, Ticks, and Tissue Factor

This editorial was originally published in *Journal of Thrombosis and Haemostasis*.

McEachron T, Mackman N. Tumors, Ticks and Tissue Factor. *Journal of Thrombosis and Haemostasis*. 2009; 7(11):1852-1854.

More than a century ago, Armand Trousseau first described an association between cancer and the coagulation system[1,2]. Later it was discovered that tumor cells release procoagulant microvesicles (often referred to as microparticles) into the culture medium that may be responsible for activation of the coagulation system[3]. The procoagulant protein tissue factor (TF) is expressed by a variety of tumors. Importantly, levels of TF expression increase with advanced cancer stage and high levels are associated with an increased mortality[4–7]. In glioblastoma cells, TF expression is induced by hypoxia and activation of the epidermal growth factor receptor[8,9]. One reason for the increased mortality may be that cancer patients have a high rate of venous thromboembolism. For instance, 11.1% of brain cancer patients have a thrombotic event within 1 year of diagnosis[10]. Indeed, tumor cells release TF-positive microparticles into the blood in mouse models and in cancer patients, and these microparticles may be responsible for triggering venous thrombosis[11–14]. Activation of coagulation by tumor cell TF also enhances pulmonary metastasis in a fibrin-dependent manner[15,16]. Finally, tumor cell TF enhances tumor growth and angiogenesis[6,17]. An earlier study found that overexpression of TF in Meth-A sarcoma cells increased tumor growth and angiogenesis in mice[18]. More recently, Rak and colleagues[19] showed that a selective decrease in TF expression reduced the growth of human colorectal cancer cells and angiogenesis in severe combined immunodeficiency mice.

Ixolaris is a tick salivary protein that has two Kunitz-like domains that are similar to the Kunitz domains found in tissue factor pathway inhibitor. In this issue of the *Journal of Thrombosis and Haemostasis*, Carneiro-Lobo *et al.*[20] demonstrate that inhibition of the TF–factor (F)VIIa complex with Ixolaris decreases the growth of human glioblastoma tumors (U87-MG) in nude mice without increasing bleeding[20]. Moreover, the inhibitor reduced

vascular endothelial growth factor (VEGF) expression and angiogenesis. There are two limitations of the study. First, U87-MG cells were injected subcutaneously rather than intracranially. Orthotopic xenografts are more physiological models of tumorigenesis, and in the case of gliomas it is unlikely that systemic administration of Ixolaris would gain access to the brain. Second, Ixolaris inhibits both the TF–FVIIa complex and activation of FX by the intrinsic tenase complex. Therefore, it is unclear if the effects of Ixolaris are as a result of inhibition of the TF–FVIIa complex and/or a reduction in levels of the downstream coagulation proteases FXa and thrombin (Figure A2.1).

Most of the *in vitro* studies on TF–FVIIa signaling have been performed using a human keratinocyte cell line and MDA-MB-231 human breast cancer cells[21,22]. In MDA-MB-231 cells, the TF–FVIIa complex activates protease-activated receptor-2 (PAR-2) and induces the expression of several pro-angiogenic mediators, such as VEGF, interleukin-8 (IL-8) and chemokine (C-X-C motif) ligand 1 (CXCL1)[22–24]. This led to the notion that TF expression by tumor cells enhances tumor growth *in vivo* by activation of PAR-2 (Figure A2.1). As noted by Carneiro-Lobo *et al.*[20], MDA-MB-231 cells express very high levels of TF in comparison to U87-MG glioblastoma cells. We analyzed TF expression in an array database[25] and found that MDA-MB-231 cells express much higher levels of TF than 99 different primary breast tumor samples of varying stages and grades (T. McEachron, F. Church, N. Mackman, unpublished data). The results indicate that MDA-MB-231 cells may not be the best breast tumor model for studying TF-related signaling events.

The hypothesis that tumor cell TF enhances tumor growth *in vivo* has been tested in a variety of mouse models. One study showed that inhibition of the TF–FVIIa complex with NAPc2, a nematode anticoagulant protein, decreased tumor growth and angiogenesis of B16

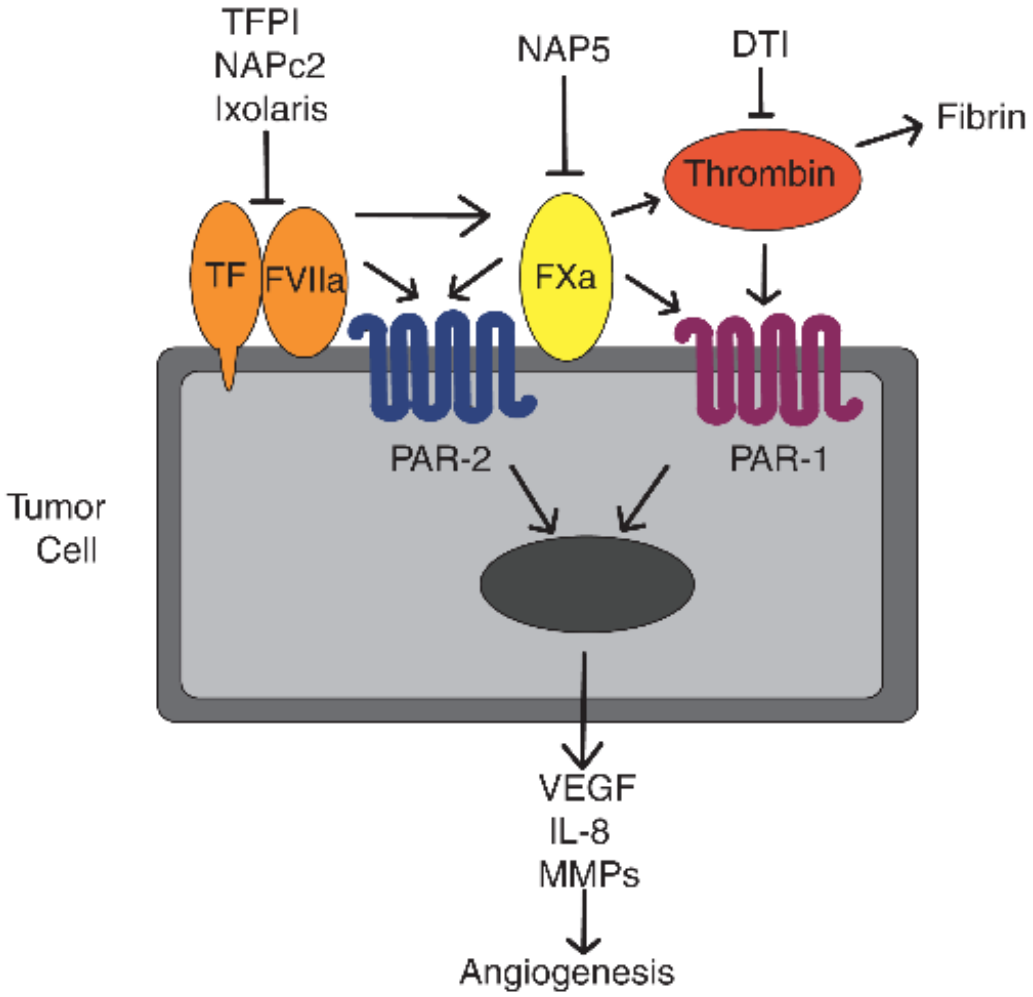

Figure A2.1. Contribution of tissue factor (TF), coagulation proteases and protease-activated receptors (PARs) to tumor angiogenesis. Formation of the TF–factor (F)VIIa complex on the surface of tumor cells activates the coagulation system. Cleavage of PAR-2 by FVIIa or FXa induces the expression of various pro-angiogenic proteins, including vascular endothelial growth factor (VEGF), interleukin-8 (IL-8) and matrix metalloproteinases (MMPs). Activation of PAR-1 by thrombin or FXa induces a similar set of genes. Various anticoagulants target different proteases of the coagulation cascade, such as direct thrombin inhibitors (DTIs).

melanoma cells and Lewis lung carcinoma cells[26]. NAPc2 also inhibited the growth of colorectal tumors in mice[27]. In contrast, specific inhibition of FXa using the nematode anticoagulant protein NAP5 did not reduce tumor growth[26]. In other studies, a humanized anti-TF antibody called CNTO859 inhibited growth of MDA-MB-231 tumors and human epithelial tumors in immunodeficient mice[9,28]. These studies demonstrate that inhibition of TF reduces tumor growth in a variety of mouse models.

Other studies have focused on the mechanism by which TF contributes to tumor growth. Importantly, a monoclonal antibody called 10H10, which inhibits TF–FVIIa signaling without affecting its procoagulant activity, reduced tumor growth and angiogenesis of both MDA- MB-231 and melanoma (m24Met cells)[29]. Moreover, inhibition of PAR-2 but not PAR-1 decreased the growth of the MDA-MB-231 xenografts[29]. Finally, in a genetically engineered mouse model of adenocarcinoma, tumors developed more slowly in PAR-2−/− mice compared with tumors in either wild-type mice or PAR-1−/− mice[30]. However, tumors isolated from PAR-2−/− mice exhibited the same growth rate in wild-type mice as those isolated from control mice[30]. Although these studies support a role of TF and PAR-2 in tumor growth in different mouse models, further studies are required to determine if the TF–FVIIa–PAR-2 signaling pathway is necessary for the growth of a wide variety of tumor types.

The paper by Carneiro-Lobo used a human glioblastoma tumor cell line[20]. Importantly, several studies have shown that thrombin plays a prominent role in the growth of gliomas by increasing VEGF expression in both human and rat glioma cell lines[31,32]. In addition, intracerebral infusion of argatroban, a specific thrombin inhibitor, reduced tumor growth in a C6 glioma model[33]. These results indicate that thrombin, possibly via PAR-1 signaling, plays a role in the growth of gliomas *in vivo* (Figure A2.1).

It is somewhat surprising that there are few reports of bleeding in studies using anticoagulants to treat tumor-bearing mice. The one exception is that specific inhibition of FXa using the anticoagulant protein NAP5 resulted in high mortality rates as a result of intraperitoneal hemhorrage[26]. One possibility is that tumor-bearing mice are hyper-coagulable because of the presence of TF-positive microparticles in the blood[11,12,19] (J.-G. Wang, T. McEachron, N. Mackman, unpublished data). These procoagulant microparticles may prevent hemorrhage in anticoagulated mice and explain the low incidence of bleeding in these studies.

Inhibiting the TF–FVIIa complex is a potential therapeutic approach to treat multiple solid tumor types. Additional benefits of this approach would be a reduction in metastasis and thrombosis. However, the greatest challenge to targeting TF is to find an efficacious dose of inhibitor that does not cause bleeding in cancer patients.

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